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Analysis of early bovine embryogenesis after in vitro and in vivo oocyte maturation by time-lapse imaging and 3-D confocal microscopy

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LIST OF ABBREVIATIONS

BSA	bovine serum albumin
CLSM	confocal laser scanning microscope
COC	cumulus oocyte complex
FSH	follicle stimulating hormone
GnRH	gonadotropin releasing hormone
h	hours
hpi	hours post insemination
ICM	inner cell mass
ICSI	intracytoplasmatic sperm injection
IVC	in vitro culture
IVF	in vitro fertilization
IVM	in vitro maturation
IVP	in vitro production
LED	light emitting diode
mg	milligram
µL	microlitre
mL	millilitre
MZT	maternal zygotic transition
OCS	oestrus cow serum
OPU	ovum pick up
$PGF_{2\alpha}$	prostaglandin 2α
PRID	progesterone releasing intravaginal device
SCNT	somatic cell nuclear transfer
SD	standard deviation
SOF	synthetic oviductal fluid
TE	throphectoderm
UFO	unfertilized oocyte
WOW	well-of-the-well

I. INTRODUCTION

The in vitro production of embryos in several mammalian species, including human, is nowadays routine laboratory work. In humans, assisted reproduction is used to overcome remaining infertility in couples with an unfulfilled wish to have children. In farm animals, beside the production of important breeding animals, in vitro produced embryos often serve as the basis for fundamental research of embryonic development. Due to ethical reasons and a strict German embryo protection law, the production and use of human embryos for the purpose of research is not possible. Therefore cattle have been used as suitable animal models to investigate the reasons for infertility in women since decades.

Embryo transfer in usually uniparous mammals like cattle and humans brings somehow a quandary with it. On the one hand, the transfer of more than one embryo increases the pregnancy rate; on the other hand, multiple deliveries are not desired and should be prevented. To increase the success rate of embryo transfers but to decrease the rate of multiple pregnancies at the same time, the selection and transfer of a single embryo is aspired. To select embryos with a high chance to develop to the blastocyst stage, many variables have been tested. Recently, time lapse imaging of embryos was found to be a useful tool to predict the development capacity of embryos (Wong et al., 2010). Thereby the fate of an embryo seems to be determined very early during development, namely during the first and second cleavage.

In order to search for the reasons and mechanisms that determine failure or success in early embryogenesis, in this experiment a time-lapse monitoring system was established for bovine in vitro produced embryos. By analyzing the timing and morphology of the cleavage events, new time values for the prediction of the developmental capacity of an embryo to the blastocyst stage were investigated. As there are references from further experiments, e.g. that the developmental capacity of an embryo is already influenced before fertilization, namely during oocyte maturation, the effect of in vitro versus in vivo maturation on the developmental morphokinetics and the developmental competence was included into the time-lapse analyses. This is becoming even more important, since in vitro maturation is also being performed in human oocytes, although in vitro maturation could have a negative influence on subsequent embryo

development. To get further insight into the process of early mitotic cleavage on chromosomal level, the information collected by time-lapse imaging was used to select embryos for analyses with a confocal laser scanning microscope.

II. REVIEW OF LITERATURE

1. Non-invasive time-lapse monitoring of embryos in different mammalian species

1.1. Historical progress

To study early embryonic development, the documentation of early cleavages from the zygote to the blastocyst stage by cinematography has become an essential tool. Lewis *et al.* (1929) were the first to observe the cleavage of rabbit embryos in vitro with a cinematographic film. Since in vitro culture systems and in vitro embryo production were in their infancy, their studies were limited to embryos flushed at different time points after mating female rabbits. Nevertheless, these authors described parameters that are still the most important criteria of live embryo observation, like timing of the development of the zygote to the expanded blastocyst stage and embryo morphology. Thirtyeight years later, when in vitro culture had been improved, further live observations of hatching mouse blastocysts were conducted (Cole, 1967). In this experiment, a closed gas-flushed culture chamber under a microscope was used to enable continuous time-lapse cinematography under optimal culture conditions. Expansion and hatching in vitro were also studied in normal and frozen-thawed bovine blastocysts (Massip and Mulnard, 1980). Until then, in vitro studies of early embryogenesis were confined to embryos that were flushed from the uterus of female animals. With the new approaches of in vitro fertilization (Brackett et al., 1982) and subsequently in vitro maturation bovine embryos could be produced easily in a high number. The cleavage pattern of in vitro produced embryos was studied by various authors (Sirard and Lambert, 1985, Plante and King, 1992, Van Soom et al., 1992), who conducted the evaluation at different time points, yet under a stereo microscope outside the incubator. In addition to the morphological description of cleavage events, time curves for different stages were published and a correlation between timing of the first cleavage and the chance to reach the morula (Van Soom et al., 1992) or the blastocyst stage (Plante and King, 1992) was found.

The first continuous cinematography without moving the culture dish out of the incubator allowed an undisturbed and more precise determination of cleavage kinetics and a selection of the embryos with a high development capacity became

possible (Grisart et al., 1994). When the timing of the first cleavage was used as a selection criterion for human embryos, the transfer of early cleaving embryos resulted in a twofold higher pregnancy rate than the transfer of their later cleaving counter partners (Shoukir et al., 1997). In contrast, Lonergan *et al.* (1999) could not find any difference in pregnancy rate after transfer of early and late cleaving bovine embryos. However, early first cleavage was found to correlate with cryosurvival (Dinnyes et al., 1999). Time-lapse microscopy was additionally used to compare the effects of different culture media on the development kinetics of bovine embryos (Holm et al., 2002, Yoshioka et al., 2000).

For cinematography, embryos were cultured in groups in petri dishes or 4-well-dishes, while some embryos had to be excluded from the data set since they moved out of the field of view or were overlaid by other embryos (Grisart et al., 1994, Holm et al., 1998, Holm et al., 2002). This disadvantage should be prevented due to the usage of a special microwell culture system. Sugimura *et al.* (2010) used the well-of-the-well (WOW) system for time-lapse monitoring of bovine embryos and found no negative effects on their development capacity. Furthermore, they observed a higher pregnancy rate after transferring embryos cultured in WOW dishes. The timing of the first cleavage did not correlate with the conception rate while the morphology of the first cleavage influenced transfer outcomes. Special devices consisting of microscopes inside an incubator simplified the evaluation and selection of single embryos especially in human reproduction medicine, where a very limited number of embryos is available and can be transferred (Pribenszky et al., 2010b, Meseguer et al., 2012). To detect the most effective parameters for early embryo selection in humans, a combination of kinetic and morphological parameters of early embryo cleavage brought the highest accuracy in prediction of blastocyst (Wong et al., 2010) or pregnancy rate (Meseguer et al., 2012). With similar algorithms Sugimura *et al.* (2012) could predict a successful embryo transfer of bovine blastocysts with 78.9% specificity. Nevertheless, these studies are based on retrospective data analyses and, especially in bovine embryo transfer, the practical efficiency of embryo selection based on time-lapse monitoring needs further investigations.

1.2. Advantages of compact embryo monitoring systems combined with a well-of-the-well culture system

Observing embryos with a device that combines a gas-flushed culture system and a camera for image acquisition provides large benefits. Newer purpose-built time-lapse microscopes are placed inside the incubator and can be controlled from outside.

Van Soom *et al.* (1992) described the disadvantages of the conventional observation method as: "They [the embryos] probably underwent the third cleavage division between observations." and "In our study, embryo evaluation at rather short intervals (6 to 12 hours) may have caused a changing environment and an additional delay in development at the 8- to 16-cell stage." Therefore a compromise had to be made between observing the embryos as frequent as possible to miss no cleavage event and observing them as rarely as possible to not disturb their development due to variations in temperature, humidity, pH or light exposure. In contrast, compact embryo monitoring systems allow a continuous evaluation of embryos without disturbing their development by taking them out of the incubator.

According to Pribenszky *et al.* (2010b) who used a light emitting diode (LED) light source in their camera system, the embryos were exposed to one-tenth of the total light energy compared to routine evaluation under a light microscope after taking them out of the incubator. In a study about the effect of light exposure to hamster and mouse zygotes, warm white light was least affecting blastocyst rate, apoptosis rate, production of reactive oxygen species and postimplantation development compared to cool white light and sunlight (Takenaka et al., 2007). Current systems include LED light sources with wavelengths of 550 to 635 nm, which is in conformity with the range of wavelength of warm white light which Takenaka and co-workers (2007) used in their experiment. Furthermore, light exposure time per image ranges from 0.3 to 5.0 seconds and images are taken up to every 5 minutes, while the camera and the light source are switched of in-between completely to avoid electromagnetic disturbance (Pribenszky et al., 2010a, Meseguer et al., 2011).

To overcome the conflict of low blastocyst rate in single embryo culture on the one hand and the difficulty of definite embryo identification on the other hand a special well-of-the-well (WOW) culture system was developed (Vajta et al., 2000). Due to the usage of these WOW dishes, the development kinetics of

several individual embryos can be followed easily at the same time since they are kept in their wells and cannot leave their position. Several studies investigated the suitability of the WOW culture of embryos of different species. No negative impact on the development capacity and embryo quality in terms of cell count of TE and ICM (Hoelker et al., 2010, Vajta et al., 2000, Sugimura et al., 2010) or apoptotic cells (Hoelker et al., 2010, Sugimura et al., 2010) was observed. Moreover, blastocyst rate (Hoelker et al., 2010, Pribenszky et al., 2010a, Vajta et al., 2000, Sugimura et al., 2010) and pregnancy rate after transfer (Sugimura et al., 2010) were even increased after culture in WOW dishes compared to control groups. Differences in gene expression levels between embryos cultured in a WOW system and a control group were observed, while it is not clear whether this indicates a negative or positive effect on embryo quality (Hoelker et al., 2009). The beneficial effect of the WOW system on embryo development is strongly hypothesized due to the micro-environment provided by the small volume of the wells and the macro-environment given through the culture drop overlaying and connecting all WOWs. According to Vajta (2000) positive auto- and paracrine factors like insulin, insulin-like growth factor I and II (IGF-I and IGF-II), platelet activating factor (PAF) or epidermal growth factor (EGF) accumulate around the embryo and compensate the low embryo number. The macro-environment ensures sufficient supply with nutrients for embryonic growth as well as the dilution of toxic products that accumulate during embryo metabolism.

Special user software allows capturing images with settings for light intensity and exposure time, different focal planes, time of observation, time between image acquisitions and includes storage and creation of time-lapse videos. To facilitate the evaluation of the time-lapse videos the software offers various features. The user can select a single embryo and follow the development by zooming on it and marking the cleavage divisions and other notable events on a time line to receive a developmental time profile of every embryo.

1.3. Parameters predicting embryo development potential

The time-lapse monitoring is a non-invasive method that allows following embryonic development in detail and thus makes it possible to conduct basic research of the morphology and timing of early mitotic cleavage events in different mammalian species. Van Soom et al. (1992) were among the first finding

a relationship between the timing of the first cleavage and following compaction to the morula stage. Subsequently several authors reported about the predictive value of morphology and timing of early cleavage events regarding embryo quality, developmental competence and blastocyst rate or pregnancy outcome (van Soom et al., 1997, Holm et al., 1998, Lonergan et al., 1999, Sugimura et al., 2010, Dang-Nguyen et al., 2010, Sugimura et al., 2012). In human assisted reproduction, time-lapse monitoring is nowadays a well-established non-invasive tool to select high quality embryos for transfer (Pribenszky et al., 2010b, Wong et al., 2010, Hlinka et al., 2012).

1.3.1. Nomenclature

Unfortunately there is no consistent nomenclature for embryonic cleavage events from the zygote to the morula stage. Especially the term “cell cycle” is difficult to define since the length of the cell cycle of different blastomeres in one embryo might differ by hours (Holm et al., 1998). Thus from the four cell stage on the embryo runs through stages where the individual blastomeres are actually in different cell cycles due to their asynchronicity. Following the designation “cell cycle” will not be used to describe the status of the whole embryo. To avoid misconceptions a system for designations of cleavage events based on the suggestions of Kirkegaard et al. (2012b) is used following in this work (see III.2.4.1). Since human embryos are more translucent, the time when the two daughter cells are separated can be delineated (Kirkegaard et al., 2012a). This is difficult in cattle embryos due to their high cytoplasmic lipid content. Therefore it is more common in bovine embryos to record the onset of a cleavage. Accordingly, the timing of the first cleavage is defined as the time span post insemination until the onset of the first cytokinesis can be identified (Sugimura et al., 2012). Different numeration of the cleavage events might also cause confusion. Thus in bovine embryos the second cleavage includes the cleavage to the 3- and 4-cell stage and following the third cleavage is the cleavage into 5- to 8-cell stages. In contrast in human embryos the second cleavage usually means division into the 3-cell stage, whereby the third cleavage results into the four cell stage.

1.3.2. Timing of early cleavage as a predictive parameter for blastocyst formation and pregnancy success

The first authors who observed an influence of the timing of the 2-cell stage on further in vitro development did not use time-lapse imaging, but conventional evaluation of the embryos at different time points. As mentioned before, Van Soom et al. (1992) established the theory of the relation between early cleavage and the development to the morula stage. Five years later they expanded this thesis regarding blastocyst formation and hatching rate, and found "embryos which had reached at least the 2-cell stage at 30 hours post-insemination (hpi), at least the 8-cell stage at 48 hpi, and the 16-cell stage at 72 hpi, had a significantly higher developmental capacity than embryos exhibiting another cleavage pattern. van Soom et al. (1997) designated such embryos as "ideal cleaving embryos". Several authors confirmed these findings through conventional observation and reported a decreasing blastocyst rate when timing of the first cleavage is increased (Lonergan et al., 1999, Dinnyes et al., 1999). Table 1 shows a summary of time-lapse studies of bovine embryos regarding the timing of the first, second and third cleavage and cell cycle length. Even though only experiments with standard IVP are listed, it is difficult to compare the results due to different definitions of cleavage events and inconsistent nomenclature. For appearance of the 2-cell stage, the 3- to 4-cell stage and the 5- to 8-cell stage Grisart et al. (1994) found a positive correlation between the timing and the blastocyst rate at day 8. Furthermore they described a time interval during the 3rd cell cycle, where the embryos seem to rest. When this "lag phase" occurred early in the 3rd cell cycle it correlated with low developmental competence. Holm et al. (1998) reported a significantly increased length of the first (respectively duration of the 1-cell stage) and second "cell cycle" (respectively the duration of the 2-cell stage) in embryos that failed to reach the morula or the blastocyst stage. Duration of cell stages were especially prolonged shortly before the embryos arrested. Consequently, the timing of 2-cell stage, 3- to 4-cell stage and 5- to 8-cell stage were also prolonged in non-viable embryos and thus could predict development to the blastocyst stage. These findings in the effect of timing of the first, second and third cleavage were confirmed by Somfai et al. (2010) in their time-lapse study. The same effects of cleavage timing and duration were observed in mice (Pribenszky et al., 2010a). Although embryo transfer is the most meaningful evidence to check embryo quality, there are only two time-lapse studies (Sugimura et al., 2010, 2012) and

one study with conventional observation (Lonergan et al., 1999) with regard to the timing of the first cleavage as predictive value not only for the blastocyst rate but also for the pregnancy rate after embryo transfer. When Lonergan (1999) compared early (first cleavage < 30 hpi) and late (first cleavage between 30 and 36 hpi) cleaving embryos, he found no difference in the rate of successful transfers between both groups. In two time-lapse studies Sugimura et al. (2010) did not select embryos for transfer according to their timing of the first cleavage, but choose blastocysts of good quality based on morphological criteria according to the recommendations of the International Embryo Transfer Society. Retrospective logistic regression analysis showed that the duration of the first cleavage as the only selection criterion is not effective as a predictive tool for successful embryo transfer neither than the pattern the first cleavage. Also in human assisted reproduction, timing of the first cleavage event for embryo selection is topical theme at present. Due to the lower lipid concentration in the cytoplasm, human embryos appear more translucent under the light microscope than bovine ones, which allows the visual evaluation of further cell parameters. Wong et al. (2010) reported for human embryos a prediction of reaching the blastocyst rate with 94% sensitivity and 93% specificity by combining the parameters a) duration of the first cytokinesis, b) time interval between the end of the first mitosis and the beginning of the second mitosis and c) time between the second (respectively 2nd cleavage 1) and third mitotic cleavage (respectively 2nd cleavage 2) into 4-cell stage. In this study the timing of the onset of the first cleavage could not be measured, since the embryos were cryopreserved 12-18 hours post fertilization and thawed again for the experiment. Lemmen et al. (2008) observed an earlier occultation of the pronuclei and an earlier cleavage in embryos that reached more than 4-cell stage at day 2. Nevertheless timing of the first cleavage did not correlate with implantation after transfer. These findings were in conformity with Hlinka et al. (2012) who received a sensitivity of only 26% by selecting the blastocysts based on optimal cleavage timelines, meaning that only 26% of the selected blastocysts resulted in a pregnancy, which is also the average success rate of IVF cycles in humans in general. On the contrary, another study resulted in a higher pregnancy rate after transfer of embryos that were already in 2-cell stage after 25-27 hpi compared to those that had not cleaved yet (Lundin et al., 2001). In conclusion, timing of the first cleavage events correlate with the blastocyst outcome, while it is controversially discussed whether it can

predict the pregnancy rate. Since embryo transfer is not always possible many authors performed further analyses to evaluate the kinetic effect of embryo cleavage on blastocyst quality. Accordingly timing of the first cleavage had no effect on the total cell number and on the ICM cell number (van Soom et al., 1997). On the other hand, a late timing of the first cell cleavage resulted in blastocysts with a higher rate of haploidy, polyploidy or mixoploidy per blastocyst (Sugimura et al., 2012). To reach a high survival rate after cryopreservation of blastocysts, embryos of good quality should be selected. In bovine, early cleaving embryos showed a higher survival rate than late cleaving ones (Dinnyes et al., 1999). Kawakami et al. (2008) compared porcine nuclear transfer embryos with a fast, an intermediate and late timing of the first cleavage, and found the highest re-expansion rate in the intermediate group.

Table 1: Effect of time of the first three cell cycles on blastocyst or pregnancy rate (+ = significant effect, - = non-significant effect)

		Author (Grisart et al., 1994)	(Holm et al., 1998)	(Somfai et al., 2010)	Sugimura et al. (2010), Sugimura et al. (2012)
Cleavage parameter					
Time	**2-cell stage**	+	+	+	+
	3- to 4-cell stage	+	+	+	
	5- to 8-cell stage	+	+	+	
	9- to 16-cell stage	-			
Duration	**1-cell stage**		+	+	
	2-cell stage		+	+	
	3- to 4-cell stage		-	-	
	5- to 8-cell stage		-		
	9- to 16-cell stage		-		
Lag phase		+			+

1.3.3. Normal and abnormal cleavage morphology of the first and second cleavage division

Beside time profiles of the cleavage events, the morphological patterns are further criteria that can be evaluated by time-lapse monitoring. The first cleavage division into the 2-cell stage is commonly distinguished into normal and abnormal cleavage morphology. Somfai et al. (2010) described a classification that contains the most important cleavage patterns, like normal cleavage into two equally sized blastomeres without fragmentation, 2-cell stage with multiple fragments, 2-cell stage with protrusion or direct cleavage into 3- or 4-cell stage. Similar assessments are recommended for human (Balaban et al., 2011), porcine (Booth et al., 2007) and murine (Pribenszky et al., 2010a) embryos. For bovine embryos Somfai et al. (2010) found that the first cleavage appeared earlier in normal cleaving embryos, yet this did not apply to the duration of the second and third cell cycle. After an unequal cleavage, more embryos arrested before reaching the 8-cell stage compared to normal cleaving ones. All abnormal cleavage patterns showed reduced blastocyst rates, except direct cleavages into 3- or 4-cell stages. No difference in the number of ICM and TE cells was found for abnormally cleaved embryos compared to normal ones (Somfai et al., 2010). The same cleavage phenotypes are observed in human embryos, and it is recommended to evaluate the rate of fragmentation, the stage-specific cell size and the incidence of multinucleation (Balaban et al., 2011). Fragmentation also occurred in time-lapse observation of mouse embryos but since the fragments were absorbed in some cases, 36.4% of it would not have been identified by only daily observation. This becomes even more important as the fragmented embryos reached the blastocyst stage at a lower rate (Pribenszky et al., 2010a). In his “microscopic and biochemical study of fragmentation phenotypes” Van Blerkom et al. (2001) distinguished between definitive fragmentation, meaning a complete separation of the fragments from the blastomeres, and pseudo-fragments. The latter were connected to the cytoplasm of the blastomere and thus could be resorbed, while detached fragments might have occurred after the connection had been broken. Some of the fragments disappeared during cleavage, but reappeared at another place some hours later due to movements of the blastomeres within the embryo. Regarding the transfer of embryos with consisting or resorbed fragmentation, they did not observe a lower pregnancy rate compared to non-fragmented embryos. For

bovine embryos, Sugimura et al. (2012) combined prognostic factors, such as timing of the first cleavage, number of blastomeres and presence or absence of multiple fragments and received a correct prediction rate for the status pregnant or non-pregnant of 78.8%. Two years earlier they already reported of two fold higher pregnancy rate after transfer of embryos with a normal first cleavage compared to an abnormal cleavage (66.7% vs. 33.3%) (Sugimura et al., 2010). Unequal cleavage had a negative effect on the blastocyst rate in bovine (Somfai et al., 2010) and porcine embryos (Booth et al., 2007). In retrospective analyses of pregnancy outcome of transferred evenly or unevenly cleaved human 4-cell stage embryos, the uneven group resulted in significantly less pregnancies (Hardarson et al., 2001). Furthermore, the evenly cleaved embryos had an earlier first cleavage than the unevenly ones, which is also related to high developmental competence. In porcine embryos unequal first cleavage did not reduce the blastocyst rate, but embryos out of unequal cleavage had a smaller total cell number at the blastocyst stage (Dang-Nguyen et al., 2010). Regarding the direct cleavage into 3- or 4-cell stage data are only available for bovine and human embryos. Interestingly, bovine blastocysts resulting from direct cleavage from the zygote to 3- or 4-cell stage, showed an increased incidence of abnormal chromosome numbers, but had a blastocyst rate equal to normal cleaving embryos (Somfai et al., 2010). The higher rate of haploidy, polyploidy and mixoploidy in these embryos was confirmed by Sugimura et al. (2012). After transfer of directly cleaved embryos, the pregnancy rate was reduced in bovine embryos and in human embryos (Sugimura et al., 2010, Rubio et al., 2012).

1.3.4. Morphokinetics of the blastocyst stage

The timing of blastocyst formation is related to embryo morphology assessment as blastocysts collected at day 7 are of better quality than day 8 blastocysts regarding total and ICM cell number (van Soom et al., 1997), pregnancy rate (Hasler et al., 1995) and survival after cryopreservation (Dinnyes et al., 1999). Even within day 8 blastocysts, advanced stages like hatched blastocysts showed a higher cell number and less chromosomal abnormalities than early blastocysts or expanded ones (Ulloa et al., 2008). Other parameters, like blastocyst diameter and zone thickness, did not correlate with blastocyst quality or developmental potential after transfer (Hoelker et al., 2006). The process of blastocyst expansion and hatching was observed very early by time-lapse cinematography in the mouse

(Cole, 1967) and later also in bovine embryos (Massip and Mulnard, 1980). Aside from the process of continuous expansion, zona thinning and hatching, some blastocysts showed volume alterations in terms of collapsing and reexpanding. As Massip and Mulnard (1980) observed these contractions in normal and frozen-thawed blastocysts that failed to hatch, they considered this phenomenon as a negative quality parameter and assumed a deficiency in the junctions between the trophoblast cells. Slight contractions did not seem to have such a big influence as severe total collapses that interrupt the expansion and thus might disturb the hatching process. The collapsing of the blastocoel only took around 13-17 minutes, while it took 9-10 hours for the blastocyst to reexpand again (Massip et al., 1982). Niimura (2003) analyzed a large number of mouse blastocysts and tried to explain the physiological meaning of the contractions. The highest frequency of contractions was found during hatching, while the more severe the collapsing occurred, the longer it took to re-expand. Based on analysis with a scanning electron microscope, he explained a contraction as an outflow of the fluid inside the blastocoel due to inappropriate adhesions of the trophectoderm cells. As the author found a similar number of contractions in in vivo developed mouse blastocysts, he suspected moderate contractions to be normal and necessary for hatching, yet not severe contractions. Another requirement for hatching in vitro is an appropriate number of embryonic cells that allows expansion and cracking of the zona pellucida (Montag et al., 2000). In their time-lapse study of bovine embryos, Holm et al. (2002) observed an average of 6-10 contractions until hatching. Embryos that were matured, fertilized and cultured in the absence of serum showed significantly more contractions than embryos that were cultured with serum. Also, in human embryos the collapse of the blastocoel is evaluated differently. Yumoto et al. (2012) suggested a negative meaning of blastula collapse as he observed less contractions in hatching human embryos and also a faster hatching process in embryos that did not contract. Furthermore they described two types of appearance of the hatching site in the zona pellucida, namely the inward pattern due to blastocoel collapse and the outward pattern caused by blastocyst expansion. On the other hand, Kirkegaard et al. (2012b) checked whether there is more blastocyst collapse in human embryos that were biopsied at earlier stages, yet did not see any difference between the biopsy group and a control group. However, the hatching process might also have had an influence on this result, since biopsied embryos could hatch through the biopsy

hole in the zona and did not expand as much as the control embryos. Contrary results were reported by Ugajin et al. (2010) about the collapse of mouse blastocyst after prior blastomere removal.

1.3.5. Recent efforts to improve selection systems

During the last years several authors tried to improve the specificity of prognostic markers by selecting embryos using logistic regression models including several morphologic and kinetic parameters. Recently Wong et al. (2010) reached a sensitivity and specificity of over 93% for predicting the developmental competence of human zygotes to the blastocyst stage. The authors combined three parameters: a) duration of the cytokinesis that divides one cell into two blastomeres, b) time between the end of the first cleavage division and the beginning of the second one, and c) time between the second and the third mitotic cleavage. Furthermore they established a tracking algorithm for the automated analysis of time-lapse videos. Due to technical reasons, up to now cell tracking is only possible until 4-cell stage (Wong et al., 2010). Today only one commercially available software exists for automatic embryo evaluation (Eeva^{TM}). According to the software analysis parameters Wong et al. (2010) used to predict blastocyst formation the shape, number, and size of blastomeres at different time points are evaluated by tracking the cell membranes. Based on this data, the system predicts the probability of blastocyst formation (Conaghan et al. (2013). The clinical benefit not only for the prediction of the development to the blastocyst stage but also of the pregnancy rate have to be validated carefully and with appraisal of results by manual scoring systems. Promising results for clinical application were published by Conaghan et al. (2013). In their study, three skilled embryologists evaluated embryos on day 3 with only morphological parameters or with additional information given from Eeva^{tm}. The combination of manual and automatic embryo evaluation increased the specificity to predict blastocyst formation of all embryologists and further adjusted the different hit ratios in between the evaluators. Considering the kinetic history of the embryo, the application of this software could help to select the best embryos among all embryos that did not show any morphological abnormalities (Conaghan et al., 2013). Cytoplasmic movements make another parameter that was recently quantified automatically in mouse (Ajduk et al., 2011) and human embryos (Swann et al., 2012) via particle image velocimetry. These movements caused by

Ca^{2+} oscillations could be related to developmental competence of mouse zygotes (Ajduk et al., 2011), while no correlation had been found for human embryos (Swann et al., 2012). Furthermore, the method requires image acquisition every 10 seconds, imposing new requirements on embryo monitoring systems. Aside from morphokinetic parameters, non-invasive measurements of metabolic activity may improve embryo evaluation and selection. In farm animals investigations regarding glucose (Peippo et al., 2001), amino acids (Booth et al., 2007) or oxygen consumption (Sugimura et al., 2012) showed promising results. Including several kinetic and morphological factors based on time-lapse imaging and oxygen consumption at the blastocyst stage into a logistic regression model, Sugimura et al. (2012) could predict pregnancy rate after transfer of bovine blastocysts with an accuracy of 78.9%.

1.3.6. Factors influencing embryo kinetics and morphology

1.3.6.1. Culture conditions

One important parameter that might have an influence on the morphokinetics of embryonic development is the different culture condition in every laboratory. However, time-lapse monitoring is a suitable tool to observe the effect of defined substances on embryo growth by adding them to the culture media. When Peippo et al. (2001) added glucose to the culture medium for the first 24 hours, they found a relationship between the presence of glucose, the sex of the embryo and the timing of the first three cleavage divisions. As no glucose was added, the female embryos went faster through the first three cleavage divisions than male ones. In the presence of glucose the development of the female embryos was slowed down, while the male embryos cleaved earlier than without glucose. Furthermore, with the presence of glucose male embryos resulted in blastocysts more often than female ones under the same conditions. Interestingly, the embryos under time-lapse observation for the first 24 hours had a lower rate of male blastocysts compared to a control group (Peippo et al., 2001). When embryos were matured and fertilized in vivo by the group of Holm et al. (2002) they apparently had an advantage regarding their development to the blastocyst stage compared to embryos matured and fertilized in vitro. This was evident from the shorter second, third and fifth cell cycle. To check the effect of serum on embryo maturation, fertilization and culture, they created several groups with or

without serum in the different steps of in vitro production. Serum application during in vitro maturation and fertilization resulted in an increase of abnormal first cleavage divisions, yet without serum, the duration of the first and fourth cell cycle was increased. Addition of serum during culture shortened the fourth cell cycle and resulted in earlier blastulation. With intent to improve the conditions of in vitro maturation Pers-Kamczyc et al. (2010) added growth hormone to the maturation medium. They observed no effect of growth hormone on the general cleavage or blastocyst rate neither on the timing of the first cleavage. However in late cleaving embryos the hatching ability of blastocysts was increased significantly in the presence of growth hormone. The highest cell count with the lowest apoptotic index was observed in early cleaving embryos that were treated with growth hormone.

1.3.6.2. Embryo gender

Beside the culture conditions, the characteristics of the embryo itself may have an effect on developmental parameters. In terms of the embryo sex, contrary results were published from different authors. Holm et al. (1998), for example, did not see an effect of the gender on the timing of any cleavage stage from the zygote to blastocyst stage. Lonergan et al. (1999) collected groups of embryos that had run through the first cleavage until 30, 36 or 42 hpi and observed in general a higher percentage of male embryos in 2-cell stage and in blastocyst stage embryos. Nevertheless the sex ratio did not differ in between the different time groups and thus was not related to the timing of the first cleavage. Lundin et al. (2001) also could not see any difference in the sex ratio of transferred embryos that were selected based on the timing of the first cleavage. In contrast, Yadav et al. (1993) found a higher ratio of male embryos in early cleaving embryos (24-30 hpi) than in later cleaving ones. Male embryos were also found to develop earlier to the blastocyst stage than female ones, as there was a higher ratio of male embryos in the more advanced blastocyst stages, as well as in hatched and hatching ones (Avery et al., 1992, Xu et al., 1992). The same tendency was observed by some authors in human embryos, as there is a shift towards male sex after the transfer of blastocysts (Luna et al., 2007, Chang et al., 2009). In contrast, Grisart et al. (1995) reported that male embryos do not develop faster to the blastocyst stage than female ones. As mentioned before, the addition of glucose into the culture medium could delay the timing of the first cleavage of female embryos and

increase the development speed of male ones (Peippo et al., 2001). The fact that the composition of culture medium can influence the sex ratio might be explained due to physiological differences of female and male embryos (for a review, see Gardner et al. (2010)). Not only external factors but also intrinsic factors of the gametes themselves might have an impact on the sex ratio. In an experiment where four different bulls were used for IVF, three bulls showed no shift of the expected sex ratio, while one bull showed an increased percentage of male embryos in the 2-cell and morula stage (Alomar et al., 2008).

1.3.6.3. Maternal and paternal influence

When Alomar et al. (2008) used sperm of four different bulls for IVF, they observed differences in the fertilization rate, the developmental speed to the pronucleus stage, and the occurrence of polyspermic zygotes between the sires. Regarding the timing of the first cleavage, two bulls showed an earlier timing than the other ones. While there was also a difference in the timing of the appearance of the morula and blastocyst stage, the cell cycles following to the first cleavage did not differ and seemed to be under the influence of maternal genes (Alomar et al., 2008). Similar results were published earlier by Ward et al. (2001), as they observed different timing of the first cleavage in six different bulls, while the timing of the first cleavage in all groups had the known effect on the blastocyst outcome. Comizzoli et al. (2000) also found an earlier first cell cycle in embryos that came from a bull with high in vitro fertility compared to a bull that brought a low blastocyst rate. In further analyses the authors found no differences in the time of the pronucleus stage, but saw a paternal influence on the length of the G_1-phase and on the onset and length of the S-Phase and the subsequent duration of the first cell cycle. Not only paternal but also maternal parameters, like the intrinsic factors of the oocyte, are suspected to influence embryo development. For the first and second meiosis, mRNA and proteins are required, which are accumulated during oogenesis and further stored in the oocyte (Sirard, 2001, Brevini et al., 2002). Since in bovine embryos the maternal-to-zygotic transition occurs between the 8- and 16-cell stage, the early zygotic cleavages are under maternal control (Telford et al., 1990). Stability and regulation of maternal mRNA depends on their polyadenylation status. Differences in this polyadenylation were observed in embryos with an early or late first cleavage (Brevini et al., 2002). This might be an explanation for the higher developmental competence of oocytes

after in vivo maturation compared to in vitro maturation, as described by Holm et al. (2002). Therefore the source of an oocyte plays an important role in the success or failure of early embryogenesis. For example, oocytes obtained from calves showed a lower blastocyst rate as well as delayed cleavage events compared to oocytes from cows (Majerus et al., 2000). Furthermore, oocytes from calves mainly arrested before reaching the 9-cell stage. Recently Dorji et al. (2012) confirmed the different quality of oocytes from adult and prepubertal cattle as they found differences in the gene expression of immature oocytes of both age groups that even increased after maturation. Alterations in gene expression and possibly resulting differences in further development could also be measured for 2-cell stage embryos after early and late cleavage. Thus, Lonergan et al. (2000) observed a higher expression of the housekeeping genes glucose-6-phosphate dehydrogenase and hypoxanthine phosphoribosyl transferase when the first cleavage occurred at 27 and 30 hpi compared to embryos that had cleaved after 33 hpi. In addition, they examined the role of insulin-like growth factor I (IGF-I) in early embryonic development and found a decreasing level of mRNA for IGF-I with the delay of the first cleavage. Lower expression levels in late cleaving embryos were also found for other genes like growth differentiation factor 9, OCT 4, GAPDH (Gendelman et al., 2010) and Histone H3 (Fair et al., 2004). These findings suggest that decreased oocyte potential is due maternal deficiency on mRNA levels.

2. In vitro versus in vivo maturation of oocytes

2.1. Oocyte maturation in vivo

In mammals, oocytes are physiologically arrested in the prophase of meiosis I. The inhibition of the resumption of meiosis was attributed to theca cells surrounding the oocytes inside the follicle (Richard and Sirard, 1996). Consequently, isolation of the oocyte from the follicle leads to spontaneous maturation (Pincus and Enzmann, 1935). In the follicle, oocytes may stay arrested for years until luteinizing hormone (LH) surges and indicates the progression to the metaphase II (M II) stage (Gordon, 2003). This event is associated with alterations in the following cell compartments (Hyttel et al., 1986, Sirard, 2001, Gordon, 2003): The nuclear maturation includes a breakdown of the germinal vesicle, the separation of the homologous chromosomes, the extrusion of the first

polar body and the alignment of the condensed chromosomes in the equatorial plate of M II. Inside the cytoplasm, the cell organelles are rearranged, and molecular maturation includes the accumulation and storage of mRNA, which determines the developmental competence until MZT (Sirard, 2001, Brevini et al., 2002). The oocyte is embedded in the follicle until ovulation and thus the interaction between the germ cell, somatic cells, and para- and autocrine factors should not be neglected (for review see, Canipari (2000)). After ovulation, meiosis is arrested again until fertilization initiates resumption. To obtain in vivo matured oocytes at metaphase II stage, several protocols for exogenous hormonal superstimulation have been developed (Bordignon et al., 1997, Hendriksen et al., 2000, Dieleman et al., 2002, Rizos et al., 2002, Humblot et al., 2005, Faasch et al., 2009). After superstimulation with follicle stimulating hormone (FSH) and gonadotropin releasing hormone (GnRH), the oocytes were obtained by ovum pick up (OPU) shortly before ovulation. Contrary results were observed regarding the developmental competence of vivo and in vitro matured oocytes as some authors saw no difference compared to in vitro matured oocytes (Dieleman et al., 2002) while others observed an improved developmental competence after in vivo maturation (Rizos et al., 2002). Over all, the blastocyst rate seems to be higher after in vivo maturation as the common 40% blastocyst rate after in vitro maturation were overtopped. By modifying the superstimulation protocol, Blondin et al. (2002) even reached a blastocyst rate of 80% at day 8. For summary of publications see Table 2.

Table 2: Developmental competence of oocytes after in vivo maturation

Reference	Cleavage rate (%)	Blastocyst rate (%)
(Bordignon et al., 1997)	83	60
(van de Leemput et al., 1999)	86	49
(Rizos et al., 2002)	87	58
(Dieleman et al., 2002)		41
(Humblot et al., 2005)	82	58
(Blondin et al., 2002)		80

2.2. Culture systems for in vitro maturation (IVM)

The maturation process that usually occurs in the follicle can also be initiated in vitro by different culture systems. Although lots of research was done to improve *in vitro* maturation, these oocytes are still of poorer quality compared to their in vivo matured counter partners, and the blastocyst rate usually reached is only around 40% (Lonergan et al., 2003b). The tissue culture medium 199 buffered with bicarbonate or HEPES is commonly used for maturation of bovine oocytes (Gordon, 2003). Depending on different protocols, bovine serum albumin or oestrus cow serum, gonatrophins (LH and FSH), and steroids are supplemented (Gordon, 2003). It is widely known that, in vitro, cumulus cells are also necessary for oocyte growth and maturation for nutritional support and the production of various growth factors (Gordon, 2003, Canipari, 2000). To detect other factors needed for maturation, defined culture media regarding serum, gonadotrophins,

steroids, growth factors, cytokines, antibiotics, but also pH, osmolarity, and the gas phase had been tested (Gordon, 2003). The optimal maturation time ranges between 18–24 hours, since developmental competence decreases (Enright et al., 2000) and abnormal nuclear maturation increases (Nakagawa et al., 1995) with a maturation time longer than 24 hours. Efforts were expended to develop a culture system in which meiosis is inhibited after isolation of the oocyte out of the follicle (Sirard, 2001, Albuz et al., 2010). This strategy is based on results which suggest that pre-maturation before resumption of meiosis is crucial for the acquisition of developmental competence. This is indicated by the fact that oocytes recovered from medium or large follicles have a higher competence than oocytes from small follicles (Pavlok et al., 1992, Lonergan et al., 1994). When an oocyte is isolated from a small follicle and meiosis resumes, chromosomes condense and RNA synthesis is interrupted. Since the stored mRNA is actually necessary for the development until MZT, these oocytes might have a reduced chance to develop, irrespective of their further treatment for in vitro maturation (Bilodeau-Goeseels, 2012). Albuz et al. (2010) tried to optimize a maturation system that includes a pre-maturation and a maturation step. To prevent maturation, they induced high cAMP levels in the oocyte and thus reached better communication between the cumulus cells and the oocyte. After FSH addition, a more homologous maturation of all oocytes was found. Subsequently, the blastocyst rate could be increased to 69% in bovine embryos.

2.3. Oocyte competence after in vitro versus in vivo maturation

As already mentioned, the intrinsic factors of oocytes have an impact on the further developmental capacity of an embryo. This developmental competence is determined very early in oogenesis during oocyte maturation and is reduced after in vitro maturation compared to in vivo maturation. According to several authors, blastocyst rate was between 50 and 60% after in vivo maturation and subsequent in vitro fertilization (Rizos et al., 2002, Bordignon et al., 1997, van de Leemput et al., 1999, Hendriksen et al., 2000) compared to around 40% after in vitro maturation. Interestingly, Rizos et al. (2002) found the highest blastocyst rate in the in vitro group when the first cleavage took place around 30 hpi, yet found a delayed optimal timing for the first cleavage in the in vivo matured oocytes. Maturation conditions could clearly determine developmental competence in this study and thus improve the blastocyst yield and furthermore the blastocyst quality

regarding survival after cryoconservation. In a time-lapse study Holm et al. (2002) compared in vivo matured and fertilized oocytes to in vitro matured and fertilized. Unfortunately, timing of the first cleavage could not be evaluated, as the time point of ovulation was not known; yet they found the duration of the second, third and fifth cell cycle to be prolonged after in vitro maturation and fertilization. In contrast, timing of compaction, blastulation and hatching did not differ between the groups, but blastocysts derived from in vivo matured zygotes showed a smaller diameter shortly before hatching process. Aside from developmental competence, the quality of the embryos and the reduction of negative side effects from in vitro maturation are crucial for a safe clinical approach, especially in human IVP. For this purpose, animal models can be used for references. It is commonly accepted, that in vitro produced embryos are different from in vivo derived embryos regarding morphology (Massip et al., 1995, Rizos et al., 2002), ploidy (Viuff et al., 2000, Slimane et al., 2000), cell number (Viuff et al., 2000), survival of cryoconservation (Fair et al., 2004, Dieleman et al., 2002) and gene expression (reviewed by (Niemann and Wrenzycki, 2000, Gad et al., 2012)). In contrast, not enough data is available up to which point maturation can determine these alterations seen at the blastocyst stage. Watson et al. (2000) demonstrated that the maturation medium in vitro could influence oocyte mRNA levels and that these levels correlate with the capacity to develop to the blastocyst after stage. Consistent with these findings, Lonergan et al. (2003a) found a different gene expression in bovine oocytes matured in vivo compared to in vitro, what might be related to different intrinsic oocyte quality. In contrast, Knijn et al. (2002) could not find alterations in the expression of six genes when comparing different blastocysts from in vitro maturation, from pre-maturation in vivo with following in vitro maturation, or from complete in vivo maturation. During the last decade this issue was also examined in human embryos due to the low pregnancy rate after in vitro maturation compared to in vivo maturation (23% vs. 32%) (Ferraretti et al., 2013). Thus Jones et al. (2008) compared the gene expression profile of human MII stage oocytes matured in vitro to oocytes matured in vivo, yet in different stages of meiosis. They found a decreased expression of several genes that are important for protein metabolism and gene transcription in the in vitro matured oocytes. Whether this high level of mRNA is due to new synthesis or precocious polyadenylation could not be clarified in this study and needs further investigations.

3. Fluorescence microscopy of embryos

3.1. Principles of fluorescence microscopy

When a fluorescent molecule is excited by a light source, which means protons of a certain wavelength, it emits protons in turn, yet in a lower wavelength (Murphy, 2001). This emitted light can be seen as a glow against a dark background. By staining cells with fluorescent dyes, individual proteins or molecules can be visualized with a fluorescence microscope (Alberts, 2008). Three important factors for this technique are the right selection of the light source, the fluorescent dye, and the filters. The light source can either be a mercury or a xenon arc lamp with a filter providing the proper wavelength needed to excite the dye, or it can be a laser that already emits monochromatic light. Beside an exciter filter, two other filters are needed. The dichroic mirror deflects all excitation wavelengths that are reflected by the specimen back to the light source, while emitted fluorescent light passes through and is again filtered in a third emission filter (Murphy, 2001). To visualize molecules and structures in a cell, fluorescent dyes can be conjugated to a specific antibody that binds to the molecule of interest. When two dyes with a different emission wavelength are coupled to two different antibodies, staining of different molecules in the same cell is possible by just switching the filters. With the use of a primary antibody that binds to the antigen and a secondary antibody that is coupled with the dye and binds to the primary antibody, the emitted fluorescence can be amplified (Alberts, 2008).

3.2. Benefits of confocal microscopy

Fluorescence microscopy of cells has to deal with two problems in general. When fixed cells are stained with a fluorescent dye, the whole thick specimen fluoresces. An image obtained by conventional light microscopy is a combination of out-of-focus light from all planes and the light from the actual focused part. This reduces the contrast of an image. Furthermore the fluorescent light that is emitted is scattered and cannot be matched exactly to its point of origin. The deeper inside the specimen the desired structure lies, the more the light is scattered (Conchello and Lichtman, 2005). These problems have been overcome with the principles of the confocal microscope invented by Minsky (1961). The composition of a confocal microscope and the path of light can be seen in Figure 1. Light that falls through a pinhole is focused on the specimen due to a dichroic mirror and an

objective lens. Thus only a small point of the specimen is excited. The emitted fluorescent light moves through the dichroic mirror and through a pinhole that is confocal to the first, emitting one. Thus, out-of-focus light is eliminated as it cannot reach the detector. By moving the focus of the small beam across the focal planes, exact two-dimensional images can be obtained and the object is scanned (Alberts, 2008, Paddock, 2000). Based on this method, which is called optical sectioning, stacks of images can be combined to three-dimensional animations (Conchello and Lichtman, 2005).

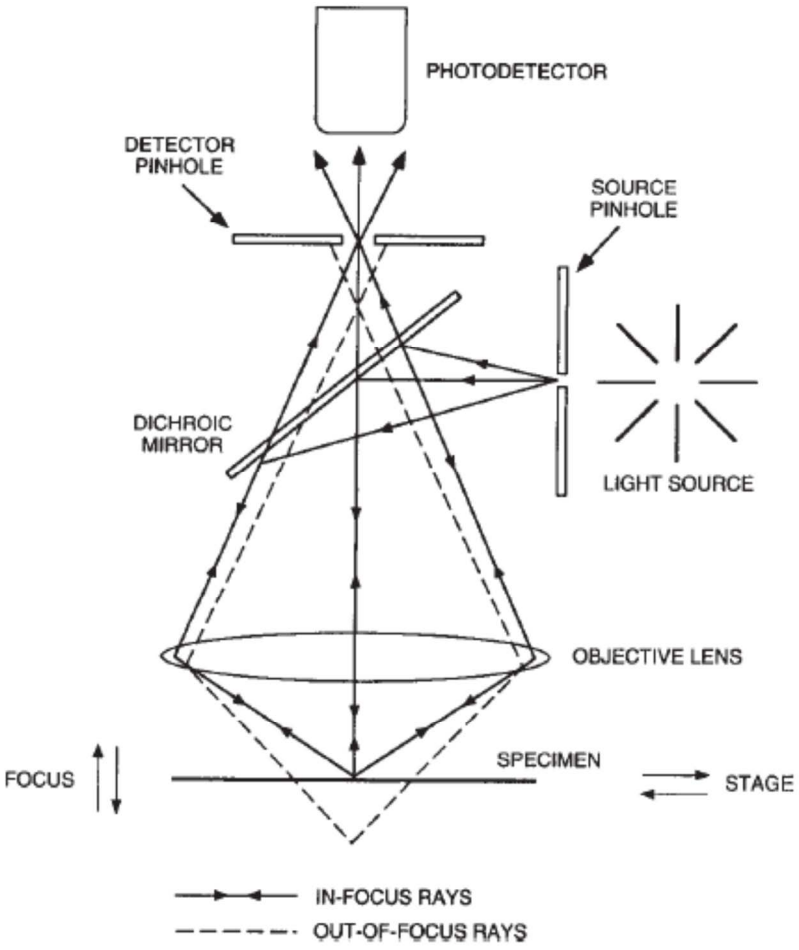


Figure 1: Path of light in a confocal microscope

Springer Paddock (2000); with kind permission from Springer Science and Business Media

3.3. Confocal laser scanning microscopy (CLSM)

A CLSM is actually a fluorescence microscope that is integrated into an electronic imaging system (Paddock, 2000). It consists of an external laser, a scan head with fluorescence filter sets, two galvanometer-driven mirrors, pinholes, a sensitive

photomultiplier tube detector (PMT), and a computer with software for image processing and display. To obtain two-dimensional pictures, the beam is moved across the specimen in a raster by two oscillating mirrors, one scanning the x-axis and the other one scanning the y-axis (Murphy, 2001). The emitted light is deflected by a dichroic mirror, while the reflected light is separated from the emitted light (Alberts, 2008). The emitted light is then focused through a barrier filter and through the second confocal pinhole, and is picked up by the PMT. The information is collected on a computer and can be processed and analyzed with special software resulting in an electronic image display (Paddock, 2000). By combining several images to a so called z-stack, three-dimensional, even rotating images can be created (Murphy, 2001).

3.4. Confocal microscopy of mitotic cell division in embryos

With the fixation of cleaving cells and following immunofluorescence staining, it was possible to observe mitotic cleavage in somatic cells (Merdes et al., 1991). Later, also meiotic and mitotic cleavage in mammalian oocytes and embryos (Schatten and Sun, 2011, Chatzimeletiou et al., 2005, Mateusen et al., 2005) were visualized with the CLSM. By using different antibodies and different dyes, several structures and processes of interest can be visualized in one cell, like mitochondria arrangement, formation of cortical granule, centrosome dynamics, calcium oscillations, spindle formation, histone distribution or chromosome segregation (Reynaud et al., 2001, Schatten and Sun, 2011, Zhang et al., 2012). Only little literature is available about confocal microscopy of bovine embryos. Long et al. (1993) examined embryos from 8 to 22 hours post insemination with an epifluorescence microscope and compared them to in vivo matured and fertilized zygotes. They found basically three groups of cleavage patterns: Oocytes that were fertilized with one sperm, polyspermic zygotes and, in both groups, failure of maternal chromatin development. From 8-12 hpi in the monospermic fertilized zygotes, they could follow the formation of the microtubule aster at the sperm tail and the decondensation of the sperm head. On the maternal side, the extrusion of the polar body and the formation of the pronucleus were visible. From 17-22 hpi they saw both pronuclei and their migration and afterwards the formation of the metaphase spindle. 29.4% of the zygotes fixated between 8-12 hpi and 66.8% of the embryos fixated between 17-22 hpi showed polyspermy. In the latter group, many various patterns of

chromatin and microtubule formations as well as different patterns of the first cell cleavage were observed. Nevertheless, the rate of polyspermy did not influence the blastocyst rate. With the use of the CLSM the quality of the pictures was dramatically improved. Thus, in human embryos the constitution of the spindle apparatus in developing and arrested embryos could be examined closer (Chatzimeletiou et al., 2005). As reasons for failure of the first cleavage, abnormal interphase nuclei and abnormal metaphase spindles were found. Micronuclei possibly result from failure in chromosome segregation, while binucleate blastomeres are caused by wrong cytokinesis. Regarding spindle abnormalities, misaligned chromosomes or chromosomes that were not even included in the spindle were observed. Furthermore, multipolar spindles were described, possibly resulting from polyspermy (Chatzimeletiou et al., 2005). Unfortunately, with these fixated embryos at different time points, it is only possible to evaluate cell structures at the status they have at the time of fixation. Since a cell cleavage is a dynamic process, it is sometimes difficult to interpret the snapshot obtained after fixation (Chatzimeletiou et al., 2005). Furthermore, permeabilization and immunostaining seem to cause artefacts like changes in the cellular ultrastructure (Schnell et al., 2012). These disadvantages could be bypassed by live-cell imaging. Recently Mizutani et al. (2012) observed mouse embryos from somatic cell nuclear transfer after injection of mRNAs that encoded two fluorescent proteins, one coupled with α-tubulin and the other one with histone H2B. With this technique, they were able to follow early cleavage events including spindle formation and chromosome segregation. Although they found abnormal chromosome segregation in 90% of the embryos, this did not affect development to the blastocyst stage. However, correct chromosome segregation until the 8-cell stage was necessary to obtain a pregnancy and cloned pups.

III. MATERIALS AND METHODS

1. Materials

1.1. Apparatuses

Primo Vision system V3	Vitrolife, Sweden
Confocal laser scanning microscope LSM 510 Meta	Zeiss, Germany
MS 5 Stereomicroscope	Leica, Germany
SSD 500 ultrasound unit with 5MHz convex array transducer	Aloka, Japan
Magnification screen Triniton KX 14 CP1	Sony, Japan
Probe holder (60 cm)	Watanabe Tecnologia Aplicada (WTA), Brazil
Follicle aspiration unit	William Cook Europe, Germany
CO_2-Incubator	Binder GmbH, Germany
CO_2-O_2-Incubator	Medcenter Einrichtungen GmbH, Germany
Megafuge 1.0 R Centrifuge	Heraeus Sepatech GmbH, Germany

1.2. Software

Primo Vision Capture Software V4	Vitrolife, Sweden
LSM Image Browser 4.2	Zeiss, Germany

1.3. Consumables

4-Well dish	Nunc, Germany
Petridish (40 mm diameter)	Nunc, Germany
Petridish (92 mm diameter)	Nunc, Germany
16-WOW culture dish	Vitrolife, Germany
Coverslips (76 x 26 mm, 0.17 ± 0.01 mm)	Hecht Assistant
Coverslips (26 x 21 mm, 0.17 ± 0.01 mm)	Hecht Assistant
Centrifuge tubes (10 ml)	Nunc, Germany
Pipette tips (250 µl, 1000 µl)	Eppendorf, Germany
Geloader ep tips (20 µl)	Eppendorf, Germany
Perforated adhesive-backed PVC-film	Mactac, USA

Sperm, Sire “Mindel” 9957197	BVN Lindenhof, Germany

1.4. Chemicals

Albumin, from bovine serum	Sigma, Germany
Aprotinin from bovine lung	Sigma, Germany
BME amino acids solution	Sigma, Germany
Deuteriumoxid	Fisher Scientific, Germany
EGTA	Sigma, Germany
Heparin	Sigma, Germany
MEM Non-essential amino acid solution	Sigma, Germany
$MgCl_2$	
NaCl 0.9 %	Sigma, Germany
Paraformaldehyde	Sigma, Germnay
Phalloidin-TRITC	
Pipes	Sigma, Germany
Polyvinylpyrrolidone	Sigma, Germany
Pyruvic acid	Sigma, Germany
Taxol	Sigma, Germany
Triton X-100	Sigma, Germany
Vectashield with DAPI	

1.5. Antibodies

Goat-anti-mouse-FITC	Dianova, Germany
Goat-anti-rabbit-Cy5	Dianova, Germany
Monoclonal mouse-anti-α-Tubulin	Sigma, Germany
Phalloidin-TRITC	Sigma, Germany
Polyclonal rabbit-anti-H3S10P	Abcam Cambridge, UK

1.6. Hormones

Follicle stimulating Hormone (FSH) 50 Units	Sioux
Luteinizing Hormone (LH) 25 Units	Sioux

1.7. Drugs

Estrumate® (Cloprostenol-Na)	Intervet, Germany
Receptal® (Buserelinacetat)	Intervet, Germany
Pluset® (Follitropin, Lutropin)	Pharmanovo, Spain
PRIDdelta® (progesterone-releasing device)	Ceva, Germany

Isocain® (Procainhydrochlorid + Epinephrin)	Selectavet, Germany

1.8. Media and solutions for in vitro production of embryos

All chemicals were used from Sigma, Germany except indicated otherwise.

Maturation medium

Modified Parker`s Medium (MPM)

Solution 1 (100 mL)	600.0 mg	Lactic acid
	100.0 mL	Aqua bidest
Solution 2 (1000 mL)	1000 mL	TCM 199 (Life Technologies, Germany)
	100.0 mg	L-gluthamine
	800.0 mg	$NaHCO_3$
	1400.0 mg	Hepes
	250.0 mg	Pyruvic acid
	1100.0 µlL	Gentamycin stock solution
Supplementation (10 ml)	5.0%	Oestrus cow serum (OCS)
	50.0 µL	(=0.025 U/mL maturation medium) FSH
	50.0 µL	(=0.0125 U/ml maturation medium) LH

Swim-up medium for sperm capacitation

Sperm TALP (500 ml)	2900.0 mg	NaCl
	1045.0 mg	$NaHCO_3$
	20.0 mg	$NaH_2PO_2H_2O$
	1190.0 mg	Hepes
	5.0 mg	Phenol red
	1825.0 µL	Na lactate sirup (60%)
	155.0 mg	$Mg_2Cl_2H_2O$
	192.0 mg	$CaCl_2H_2O$
Supplementation (10 mL)	60.0 mg	Bovine serum albumin (BSA)
	500.0 µL	Pyruvate stock

Medium for in vitro fertilization

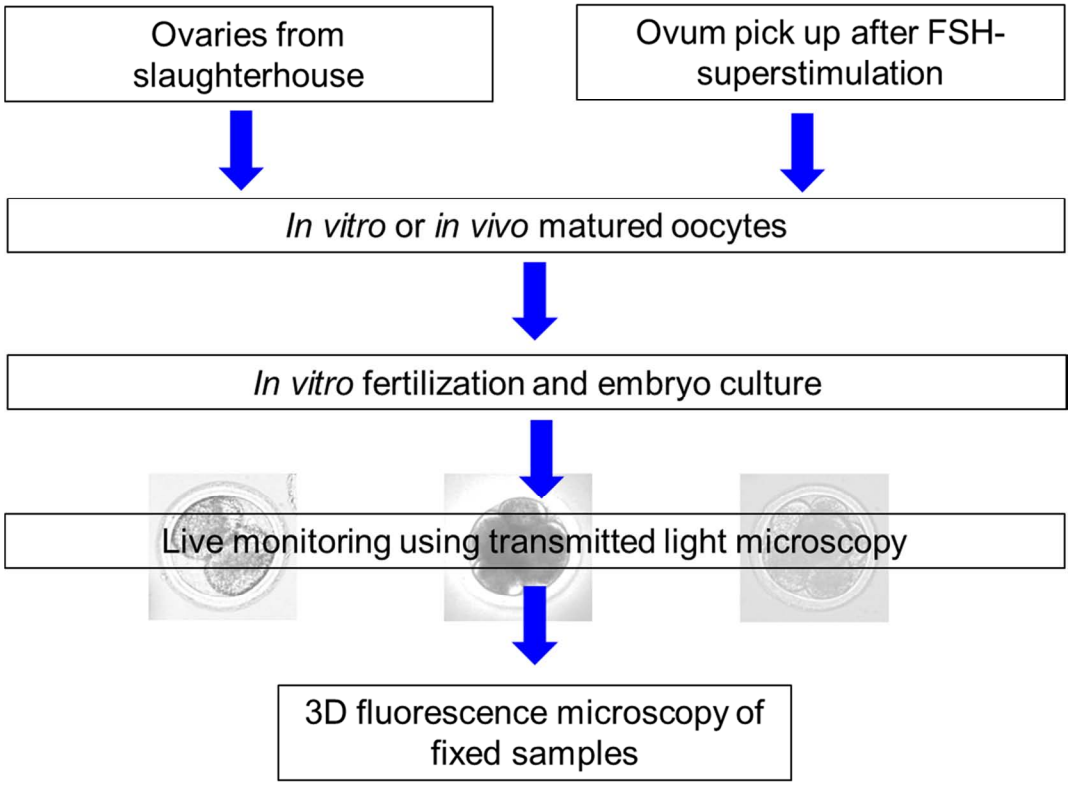
Fert TALP (500 ml)	3330.0 mg	NaCl
	117.5 mg	KCl
	1051.5 mg	$NaHCO_3$
	23.5 mg	$NaH_2PO_4H_2O$
	32.5 mg	Penicilline
	5.0 mg	Phenol red
	930.0 mg	Na lactate sirup (60%)
	50.0 mg	$MgCl_2H_2O$
	198.5 mg	$CaCl_2H_2O$
Supplementation (10 mL)	60.0 mg	Bovine serum albumin
	100.0 µL	Pyruvate stock
	250 µL	Heparin stock

Medium for in vitro culture

Synthetic oviduct fluid (SOF, 500 ml)	31460.0 mg	NaCl
	267.0 mg	KCl
	81.0 mg	KH_2PO_4
	123.9 mg	$CaCl_2H_2O$
	48.3 mg	$MgCl_2H_2O$
	1053.0 mg	$NaHCO_3$
	0.7 mg	Phenol red
	181.5 mg	Pyruvate
	2500.0 mg	L-gluthamine stock
	235.3 µL	Na lactate sirup (60%)
Supplementation (10 mL)	400 µL	BME Amino acid solution
	100 µL	MEM Amino acid solution
	5.0 %	OCS

2. Methods

2.1. Experimental approach



2.2. Live observation of bovine embryos after in vitro and in vivo maturation

2.2.1. Embryo production

2.2.1.1. Recovery of in vitro matured oocytes

COCs for in vitro maturation were obtained from ovaries collected at the Munich slaughterhouse. The ovaries were transported in PBS at 25 °C. Follicles with a diameter from 2–8 mm were aspirated with a vacuum pump (80–100 mm Hg) and follicular fluid was collected in a Falcon tube. With transferring the sediment into a Petri dish, COCs were recovered and divided into four morphological classes of quality, based on layers of cumulus cells and the color and homogeneity of the cytoplasm (Table 3). For this experiment, only COCs of class I and II were used and washed three times in MPM and cultured in 4-Well-dishes in groups of 40 oocytes in 400 µl of the same medium. Maturation time was 23 hours in an atmosphere of 5% CO_2 and at a temperature of 39°C.

Table 3: Classification of non-mature oocytes (Berg and Brem, 1989)

Class	Layers of cumulus cells	Cytoplasm
I	>5 complete layers	Homogenous color, not granulated
II	3-5 complete layers	Homogenous color
III	Few layers with gaps	Granulated and inhomogenous
IV	Denuded	Small, granulated and inhomogenous

2.2.1.2. In vivo maturation and OPU

The in vivo matured oocytes were collected by OPU of FSH-superstimulated Simmental heifers and cows. Group 1, consisting of six Simmental heifers, had two OPU-sessions in a 5-week interval. In Group 2, consisting of five heifers and 2 cows, OPU was applied three times in a 5-week interval. All animals were superstimulated according to the protocol shown in Figure 2.

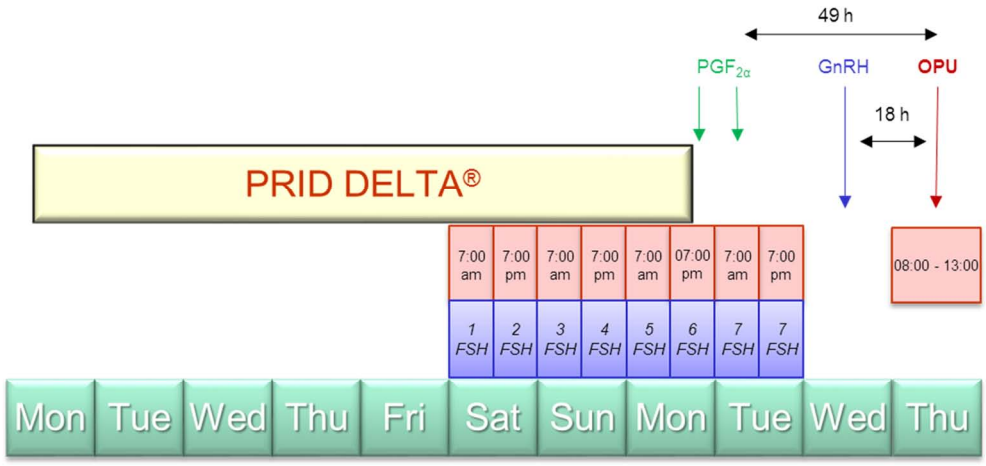


Figure 2: FSH-superstimulation protocol for the collection of *in vivo* matured oocytes

Prior to the first superstimulation, all animals were gynecologically examined. For oestrus synchronization, a progesterone releasing intravaginal device (PRID DELTA®) was inserted (day 0) and left inside for 8 days. From day 6 to day 9, FSH-injections with an interval of 12 hours were given twice daily in decreasing dosages to induce follicle growth. The daily dosage and the total amount were different for heifers and for cows (Table 4).

Table 4: FSH dosages for heifers and cows

Treatment Nr.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**Total**
FSH in ml									
Heifers	2.0	1.5	1.5	1.5	1.0	1.0	1.0	0.5	10.0
Cows	3.0	2.5	2.0	2.0	2.0	1.5	1.0	1.0	15.0

To induce the regress of a present corpus luteum, $PGF_{2\alpha}$ was injected simultaneously with the 6th and 7th FSH injection, and the PRID DELTA® was removed. 5 ml of GnRH were applied 18 hours prior to OPU in order to support follicle maturation. Follicle aspiration was performed ultrasound-guided by placing the probe holder with the transducer transvaginal in front of the cervix. To depict the follicles, the ovary was placed in front of the ultrasound probe trough rectal palpation. With an 18 Gauge needle that was guided through the probe, the follicles were punctured through the dorsal wall of the vagina. A puncture line on the ultrasound monitor helped to find the right position of the needle. The follicle fluid was aspirated by a connected vacuum system with 90–110 mmHg negative pressure and contained in a Falcon tube. The system was flushed with TCM 199, supplemented with heparin and gentamycin from time to time. All visible follicles were punctured, while the amount of follicles obtained was documented for every animal. The follicular fluid was filtered (Emcon® filter) and the filtration residue was washed out into a Petri dish. The COCs were evaluated under a stereo microscope and classified into non matured and matured oocytes. Matured oocytes were defined as oocytes surrounded by an expanded cumulus. Only matured oocytes were used for this experiment. Before fertilization, a few of the expanded cumulus cells were removed by gently pipetting the oocytes up and down in a 20 µl Eppendorf Pipette with a 200 µl tip.

2.2.2. In vitro fertilization

For in vitro fertilization, the oocytes from both maturation groups were washed three times in Fert-TALP and transferred into a 4-Well-dish, while each well contained 400 µl of Fert-TALP and 40 oocytes. For sperm capacitation, frozen semen stored at -196°C was thawed for ten seconds in a 38°C warm water bath. 10 µl of the diluted sperm were immediately layered under 1 ml of Sperm-TALP in four centrifuge plastic tubes. After incubation for 1 hour, the supernatant liquor was removed from all four tubes and centrifuged at 2400 rpm for 10 min. Since the bull had been tested and used many times for IVF, the following procedure to receive a sperm concentration of 1.000.000 sperms/ml medium was standardized. After centrifugation the supernatant medium was removed again and the remaining sperm pellet was re-suspended with 40 µl medium. For fertilization, 10 µl of the sperm suspension were pipetted in each well containing the matured oocytes and then co-incubated for 18 hours in an atmosphere of 5 % CO_2 and a temperature of 39°C.

2.2.3. In vitro culture

For observation of the embryos under the Primo Vision camera®, a special Well-of-the-Well (WOW) culture system was used (Figure 3). The dishes had a diameter of 35 mm with a bottom including 4 rows with 4 mircowells each. 16 embryos were inserted and observed at the same time, without the risk of leaving the field of view of the camera. As the mirowells were each 350 µm x 300 µm wide and 270 µm deep, 0.09µl^3 SOF per well could be filled in with a Gel loading pipette. All 16 wells were covered with a 160 µl drop of the same medium. Accordingly, the embryos were cultured in 10 µl medium per embryo. The dish was filled with mineral oil until the drop was completely covered. After the WOW dish was incubated for 1 hour, any occurring air bubbles were removed. Exactly 18 hours after sperm addition, the properly fertilized oocytes were denuded mechanically by vortexing in 1 ml ready-made SOF for 3 minutes. The zygotes were washed three times in SOF and inserted into the WOW dishes. 16 embryos were cultured per dish, while one microwell contained one single embryo. Two WOW dishes with 16 zygotes each were inserted into two Primo Vision cameras® inside the incubator and cultured at 39°C in maximum humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. After insertion, the embryos were observed for additional 168 hours under the Primio Vision camera®. To test the suitability of

the WOW culture system, a control group of 16 embryos in a 160 µl medium drop was placed in a Nunc Petri dish without mircowells. The drop was also covered with oil and cultured in the same incubator for the same period of time. Blastocyst rate was determined 186 hpi. This control groups only exists for in vitro matured oocytes, hence the number of in vivo matured oocytes was limited.

Figure 3: Primo Vision® live monitoring system for embryos
(Pictures from the Primo Vision manual)

2.3. Embryo monitoring system

For time-lapse imaging the Primo Vision® embryo monitoring system was used. Two microscopes were placed inside the incubator and cable connected to a controller station outside the incubator. A Primo Vision microscope consisted of a waterproof housing protecting the optical system and the camera, a lamp console including a green LED light source, and a dish holder designed for the WOW Petri dishes. The whole electrical supply and also the LED lamp were only switched on during placing the dish into the right position, typing the project settings, and afterwards during image acquisition, to avoid embryo disturbance. The controller unit included sockets for electrical supply of the microscope units and a micro PC for storing the images. For image capture and following analyses

the PC ran special software. The embryo development could be followed and analyzed on a screen connected to the controller unit.

2.3.1. Capture software

The capturing software provided two functional modi. At first, the live modus was used to check the position of the dish and the embryos in the wells. The optimal focus plane had to be readjusted and the light intensity was set to 40 %, the exposure time was 95 ms and the gamma value was set to 1.0. After that, the scanning modus was used to enter the project ID, the time and date of fertilization, and to set additional scan parameters. The number of focal planes that were scanned was set to 11, the scan range to 200 micrometers, and the images were taken every 5 minutes for 168 hours after starting the project. Finally, the project was started and the incubator was not opened until the observation period was ended and the next embryos were inserted.

2.3.2. Analyzer software

For the analysis of the cleavage events, the Primo Vision Analyzer Software V4 was used. The images were assembled to a time-lapse movie of all 16 embryos (Figure 4). Two timelines, one for the time post fertilization and one for the time after starting the program showed the exact time in hours and minutes while watching the time-lapse video. The video could be paused, rewinded and forwarded, or slowed down at any time point. By selecting one single embryo, its enlarged images were shown in a separate window and markings for defined cleavage events were set manually. If an embryo or a part of the sequence was not evaluable due to wrong focus or air bubbles in the well, it was marked and excluded from later statistical analyses. The developmental stages that were evaluated were selected based on an embryo evaluation system shown in 2.4.1. Time marks were saved in hpi:minutes post insemination and the software created cleavage profiles in terms of graphs and tables for all embryos.

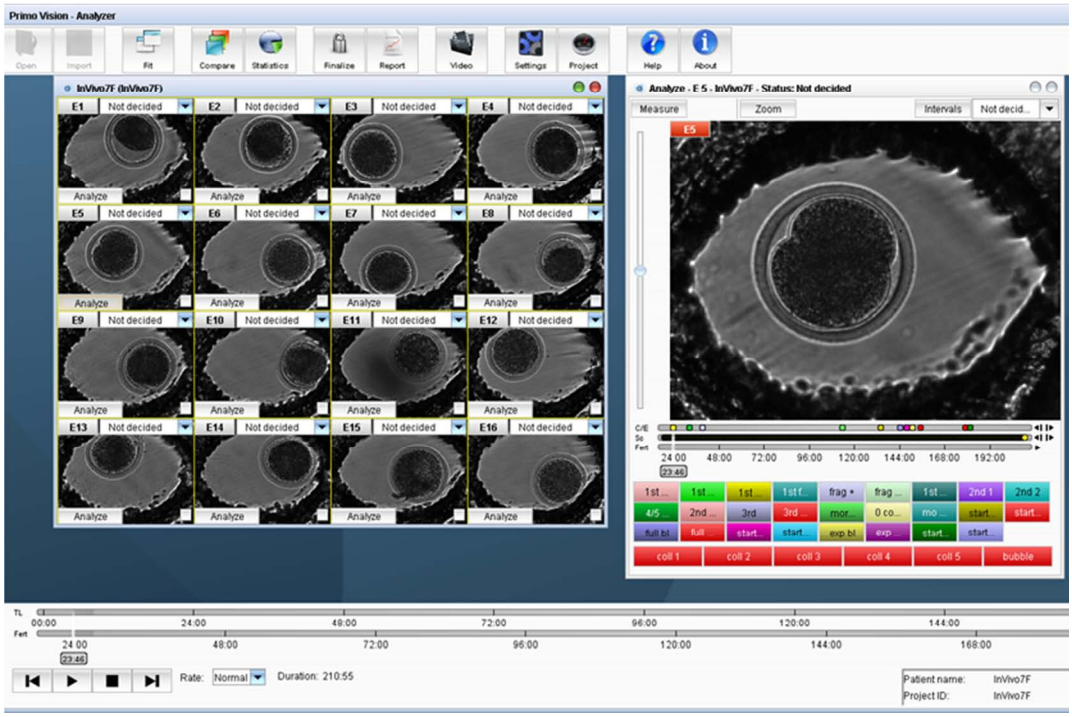


Figure 4: Embryo monitoring with the Primo Vision system

The picture shows how the software creates a time-lapse video of 16-embryos (lift side) and how an individual embryo picture can be enlarged for analysis (right side).

2.4. Data analyses

The time table for every project was exported to another computer and saved in a copious excel table. For statistical analysis the unit of time was converted from hpi:minutes into hours, minutes.

2.4.1. Embryo evaluation

To be able to compare the results of this study to the observations of other authors, a system for uniform nomenclature is suggested and was used for embryo evaluation in this experiment (Figure 5). The time point when the sperm was added to the matured oocytes was set as t_0. For the cleavage events the time in hours, minutes post insemination was documented by setting a mark in the Primo Vision Analyzer Software®. The cleavage events were measured with the beginning of the cleavage, meaning the first appearance of the cleavage furrow. Following events were evaluated: cleavage into 2-cell stage as the first cleavage (t_1), cleavage into the 3-cell stage as the first part of the second cleavage (t_{21}), cleavage into the 4-cell stage as the second part of the second cleavage (t_{22}) and cleavage into the 5-cell stage as the beginning of the third cleavage (t_3). The timing of a cleavage has to be distinguished from the duration of a cleavage cycle

that includes the developmentally consistent cleavage of each blastomere until the number of blastomeres is doubled. Thus the first cleavage cycle results into the 2-cell stage, the second cleavage cycle into the 4-cell stage and the third cleavage cycle in 8-cell stage embryos. The synchronicity within a cell cycle is defined as the time difference between the cleavage of the first and the last blastomere within this cell cycle. Regarding the first cleavage, four morphological types for the first cleavage could be observed: normal cleavage, cleavage into two unequally sized blastomeres, direct cleavage into the 3- or 4-cell stage and cleavage into two blastomeres but with additional fragments. Although the direct first cleavage resulted into three or four cells, it was treated as the first cleavage and its timing was documented as t_1. Accordingly, the second cleavage that followed onto a direct first cleavage resulted into a 4- or 5-cell stage and was documented as t_{21}. The data for t_{22} are missing in this case.

Based on the measured cleavage time points, further variables could be calculated. Within the second cell cycle, the duration of the two cell stage (Δ_{12}) and the duration of the three cell stage (Δ_{22}) were calculated. The latter is equal to the synchronicity of the second cleavage. The total duration of the second cleavage was accordingly the combination of the duration or the three and four cell stage (Δ_{23}) (Figure 5).

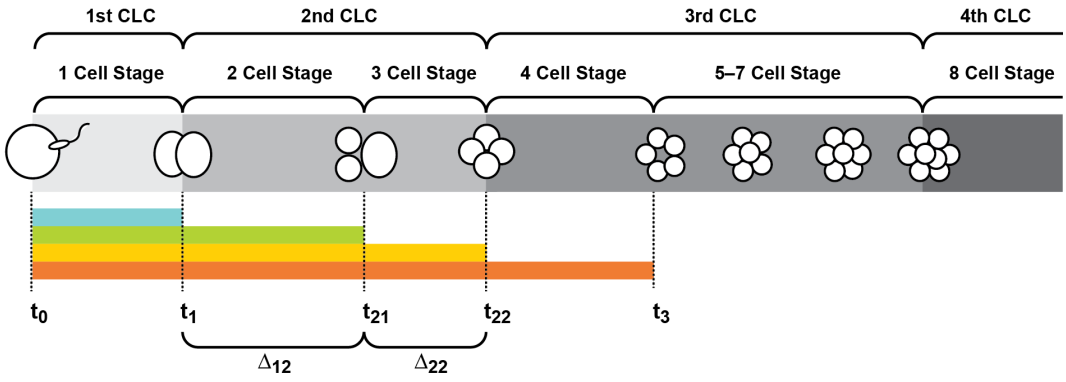


Figure 5: Suggested nomenclature of early cleavage stages in bovine embryos

Beside the timing of the early cleavages, the occurrence of the compact morula stage and five marks for blastocyst stage were evaluated. The start of blastulation was defined as the time when a small blastocoel appeared in the cytoplasm. The full blastocyst stage was reached when the blastocoel occupied the whole zona

pellucida. The start of blastulation began with the increase in zona diameter. When the zona became distinctly thinner through advanced expansion, a mark was set for the expanded blastocyst. The final stage an embryo could reach was the hatching of the blastocyst out of its zona. The beginning of this event is marked by the rupture of the zona pellucida and the protrusion of the blastocyst. Whether an embryo reached the blastocyst stage or hatched was evaluated at 186 hpi. Furthermore the number of collapses was documented for every blastocyst.

2.4.2. Statistical analysis

Statistical analyses were performed with the software SPSS 18.00. Graphs were created with GraphPad software Prism 6. Data is presented as means ± SD. Developmental rates and frequencies between the maturation groups were compared with the χ^2-test. Differences in timing of the cleavage events between the in vitro and in vivo matured oocytes were compared using the Mann-Whitney-U-test. The Pearson product-moment correlation coefficient was used to test bivariate correlations. P-values less than 0.05 were considered as significant. When the development of an embryo could not be followed over the whole period of the observation time due to images out of focus or air bubbles that covered the embryo, this embryo was marked as non-evaluable and excluded from the statistical analyses.

Logistic regression analysis was performed to find a model for the prediction of the development to the blastocyst stage, in order to be able to select embryos with a high or low developmental competence during early cleavage stages. For logistic regression analysis, the data was documented as continuous variables or dummy variables. The development to the blastocyst stage (full blastocyst stage) was set as the binary dependent variable and coded as 0 or 1. As independent variable, the type of maturation was coded as 0 or 1. Timing of cleavage events (t_x) and the durations (Δ_x) of individual cell stages or cleavages were given as continuous data in hours, minutes post insemination and the morphology of the first cleavage was converted into a dummy variable.

Dependent variable

Blastocyst stage at 186 hpi	0 = no blastocyst
	1 = blastocyst

Independent variables

Type of maturation	0 = in vitro maturation
	1 = in vivo maturation
t_1, t_1^2, t_{21}, t_{22}, t_3	Continuous variable (hpi)
Δ_{12}, Δ_{22}, Δ_{23}	Continuous variable (h)
Abnormal morphology of the first cleavage	Unequal = 1,0,0
	Direct 3-/4-cell stage = 0,1,0
	Fragmented = 0,0,1

The covariables and the logistic regression model were selected by Dr. Christiane Fuchs from the Institute of Computational Biology at the Helmholtz Zentrum Munich. As there was no value available for Δ_{22} in the case of a direct first cleavage into the 3- or 4-cell stage, the variable Δ_{22} and the variable morphology = 0,1,0 could not be in the same model. Thus two different models, each including only one of the two variables, had to be selected. The most predictive covariables and the two models were selected by stepwise backward selection based on the Akaike information criterion (AIC) with considering P values of 0.01 as significant. The predictive accuracy of the model was tested by cross validation. Following a logistic regression function was obtained based on the logistic regression model:

$$p_k = \frac{1}{1+e^{-z_k}}$$

e is the base of natural logarithm, 2.718

$$z_k = \beta_0 + \sum_{j=1}^{J} \beta_j \cdot x_{j,k}$$

with

k = individual embryo

j = number of independent variables

$x_{j,k}$ = value for the independent variable for the embryo k

β = coefficient

2.5. CLSM analyses

To get more insight into the normal and abnormal processes of mitosis on cellular and chromosomal level, fertilized in vitro matured oocytes were analyzed with a confocal laser scanning microscope (CLSM) at defined time points after fertilization. The experiment was replicated two times.

2.5.1. Embryo evaluation at different times post insemination

For each experiment, oocytes were matured and fertilized in vitro as described before. At 18 hpi the presumptive zygotes were denuded and divided into 6 groups of 40 zygotes each. Every group was cultured in an individual 4-well dish in 400 µl ready-made SOF, therewith the amount of culture medium per embryo was equal to the amount in the WOW dishes used for live observation. The groups of zygotes were fixated at 22, 24, 26, 28, 30 and 32 hpi. Based on previous observations with the time-lapse camera, mitosis was expected to take place within this time window. For fixation, one 4-well dish each was taken out the incubator at the defined time point and the zygotes were immediately transferred into 400 µl preheated Albertini-solution (Table 5). Afterwards, the zygotes were incubated for 20 minutes at 37° C, followed by two washing cycles in PBS-PVP. The fixated embryos were stained immediately or stored at 4° C in PBS-PVP until staining.

Table 5: Albertini-solution for fixation of zygotes

Pipes (0,5 M)	200 µl
$MgCl_2$ (50 mM)	100 µl
EGTA (50 mM)	50 µl
Deuteriumoxid	445 µl
10 % Paraformaldehyde	200 µl
Triton X-100	5 µl
Aprotinin	1 µl
Taxol (5 mM)	0.2 µl
Total volume	1001.2 µl

2.5.2. Fluorescence staining

To visualize the basic cell structures involved in the first cell cleavage, all fixated embryos were stained with fluorescent antibodies. For visualization of microtubules and Ser10-phosphorylated histone H3 immunofluorescence staining was performed, while microfilaments were counterstained with Phalloidin-TRITC. DNA was stained with DAPI. To allow antibody penetration, the embryos were permeabilized in 400 µl PBS-PVP with 0.5% Triton X-100 for 1 hour at room temperature. After washing in PBS-PVP three times, the zygotes were incubated in a mixture of two primary antibodies for 30 minutes at 37° C. For this purpose, mouse-anti-alpha-Tubulin and rabbit-anti-H3S10p were diluted 1:500 in PBS-PVP and drops of 50 µl were pipetted into a petri dish and overlaid with mineral oil. After three washing cycles in PBS-PVP all following steps were conducted with exclusion of light as far as possible. Following was the incubation in a mixture of the two secondary antibodies and Phalloidin-TRITC. The antibodies used were goat-anti-mouse-FITC and goat-anti-rabbit-Cy5 diluted 1:500 in PBS-PVP. Phalloidin-TRITC was added in a dilution of 1:250. The embryos were incubated again for 30 minutes at 37° C in a 50 µl drop and washed three times afterwards in PBS-PVP at room temperature. After staining, the zygotes were immediately transferred on slides or stored in PBS-PVP at 4°C and kept under the exclusion of light until the next day.

2.5.3. Embryo preparation

For analyses with the CLSM, the embryos had to be mounted in such a way that they were not compressed, but retained their original structure. For this purpose, pieces (8x15 mm) of a perforated adhesive-backed PVC-film were affixed on coverslips (76x26 mm). With a mouth pipette the embryos were transferred into a drop of Vectashield antifade solution with DAPI, and subsequently one embryo per hole was pipetted onto the coverslip under the microscope. The holes were filled up with mounting medium and the whole PVC-film piece was covered carefully with a smaller coverslip (26x21 mm). Finally the edges of the small coverslip were sealed with nail enamel. The mounted embryos were stored at 4° C under the exclusion of light until they were examined with the confocal microscope.

2.5.4. Confocal microscopic analysis

The analyses with the CLSM were performed by Dr. Felix Habermann from the Chair of Anatomy, Histology and Embryology of the LMU Munich. For the 3D-visualization of the stained embryos, a confocal laser scanning microscope (LSM 510 Meta) equipped with a 40 x PlanNeofluar (NA 1.3) oil immersion objective and the Zen 2011 7.1. control software was used. The embryos were scanned in optical sections in an interval and thickness of 1 µm (pixel size 125 x 125 µm). The individual fluorochromes were excited with different lasers lines. For the detection of the emission appropriate filters were used. The type of laser and filter used for the fluorochromes are summarized in Table 6.

Table 6: Laser-filter-fluorochrome combinations

Laser (wavelength in nm)	Emission filter (nm)	Fluorochrome
Argon (364)	385 - 470 BP	DAPI
Argon/Crypton (488)	505 – 530 BP	FITC
Helium-Neon 1 (543)	560 – 615 BP	TRITC
Helium-Neon 2 (633)	650 LP	Cy5

BP = bandpass, LP = longpass

For each sectional plane the emission of the different fluorochromes were detected consecutively and casted into 8-bit grayscale images. Special areas of interest were scanned with higher resolution. For processing and 3D visualization of large-scale image stacks the Zeiss LSM Image Browser 4.2. was used.

IV. RESULTS

1. Ovum pick up

In total 1095 follicles were punctured during five OPU sessions. As 354 oocytes were obtained, the recovery rate was 34.0 ± 14 %. 77 % (n=272) of these oocytes were surrounded by expanded cumulus cells and thus were assumed as matured and fertilizable.

2. Time-lapse observation

2.1. Developmental competence after in vitro and in vivo maturation

In total, 416 in vitro and 144 in vivo matured zygotes were observed with the time-lapse system for 186 hpi. The different stages of an embryo during its development from the zygote to the blastocyst stage can be seen in Figure 6. As 19 embryos could not be evaluated during the whole culture period, they were excluded from further statistical analyses.

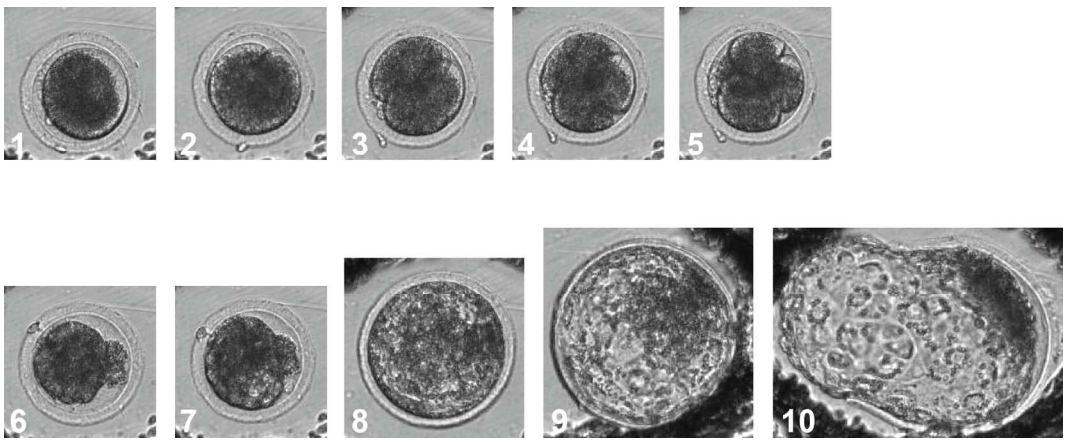


Figure 6: Embryonic development stages during time-lapse imaging

1) Fertilized oocyte
2) Beginning of the first cleavage: The cleavage furrow is visible at one side of the zygote.
3) Beginning of the second cleavage: The lower blastomere is beginning the second cleavage into the 3-cell stage
4) Completion of the second cleavage into the 4-cell stage: The second blastomere of the second cleavage has cleaved.
5) Beginning of the third cleavage: The first blastomere of the 4-cell stage starts to cleave.
6) Compact morula stage: Beside the mass of the compact morula an arrested blastomere is visible.

7) Beginning of blastulation: The blastocoel can be seen as a brightening inside the cytoplasm.
8) Blastocyst stage: The blastocoel fills the whole embryo and a TE and an ICM can be distinguished.
9) Expanded blastocyst: The blastocyst has expanded in size and the zone pellucida got thinner.
10) Hatching blastocyst: The blastocyst is hatching through a hole in the zone pellucida

The in vitro matured control group that was cultured in a normal petri dish without microwells showed a significantly lower blastocyst rate than the in vitro matured embryos that were cultured in the WOW dishes (35% vs. 44%, P=0.01). Regarding the type of maturation, developmental competence represented by the cleavage rate, the blastocyst rate and the hatching rate was high in both groups. Nevertheless, the cleavage rate was significantly higher after in vitro maturation (87.2% vs. 72.7%, P<0.001). Regarding the blastocyst rate, there was no difference between the in vitro and in vivo matured oocytes (44.0% vs. 43.4%). Although there was no significant difference in the overall blastocyst rate, the blastocyst rate of cleaved embryos was almost 10% higher for the in vivo group compared to the in vitro group (59.6% vs. 50.4%, P=0.100). There was no significant difference in the developmental competence to the blastocyst stage, yet the in vivo matured oocytes brought significantly more blastocyst hatching out of their zona pellucida compared to the in vitro matured ones (28.7% vs. 15.6%, P=0.001) (Table 7).

Table 7: Cleavage rate, blastocyst rate and hatching rate of in vitro and in vivo matured oocytes

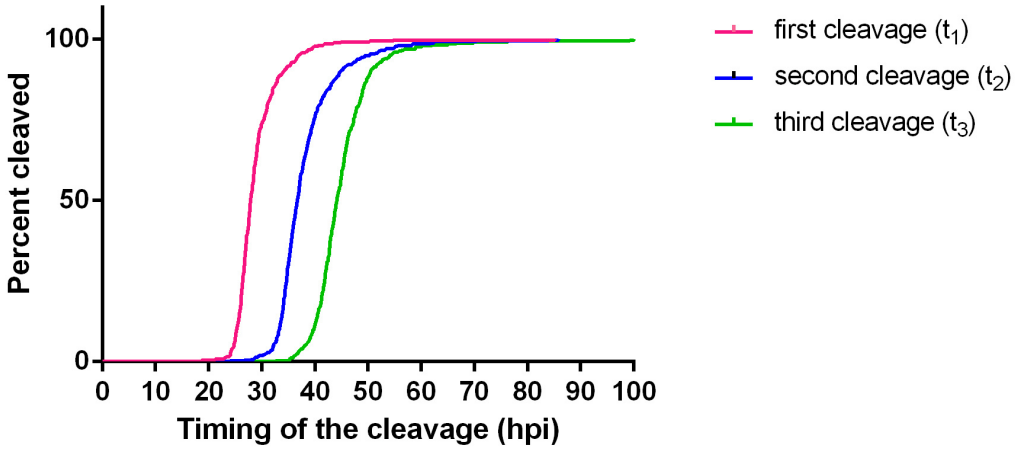
	In vitro maturation (n=398)	In vivo maturation (n=143)	P-value[a]
Cleavage rate	87.2%	72.7%	< 0.001
Blastocyst rate	44.0%	43.4%	0.899
Blastocyst rate of cleaved embryos	50.4%	59.6%	0.100
Hatching rate	15.6%	28.7%	0.001

[a] P-value of χ^2 statistic

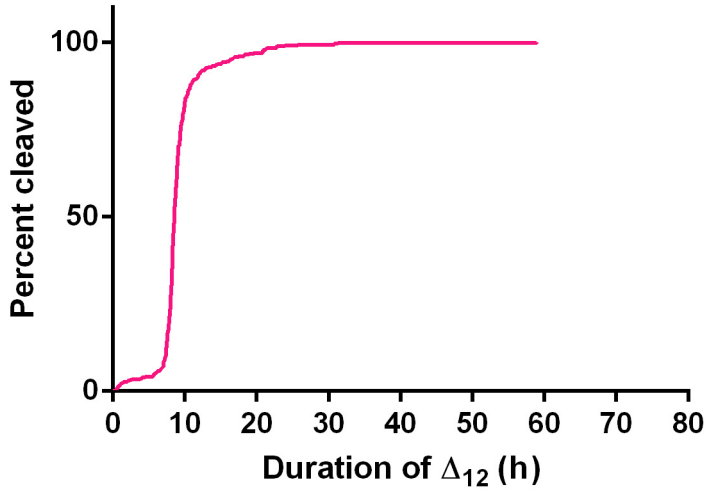
2.2. Timing of the first, second and third cleavage division

In the following, the data for the timing of the first, second and third cleavage of all observed embryos is presented. Time curves of the first three cleavages (t_1, t_2, t_3) are shown in Figure 7. All three curves have a similar shape: the time frames for the occurrence of the cleavages are very wide, but most of the embryos cleave inside a short time window, which can be seen in the steep slope of all three graphs. Accordingly, although the time window for the first cleavage ranges from 18.7 hpi to 91.5 hpi, 50% of the embryos cleave between 26.6 hpi and 30.6 hpi. The same holds true for the second and the third cleavage as the time windows range from 24.1hpi to 85.8 hpi (t_{21}) and 32.6 hpi and 122.2 hpi (t_3), but 50% of the embryos start the second cleavage between 34.6 hpi and 36.4 hpi and the third cleavage between 41.8 hpi and 44.2 hpi. As the curves continue along the X-Axis they flatten, as there are some embryos with a very late first, second and third cleavage which is illustrated by this flattening of the curves towards the end. Figure 7, B shows that the timing between the first and the second cleavage take between 8.0 and 9.4 hours in around 75% of all cleaving embryos. The time curve for Δ_{22} (Figure 7, C) also has a steep slope and shows that in 75% of the blastomere pairs of the 2-cell stage, both cleave within a time difference of one hour into the 4-cell stage.

A


Percent cleaved
100
50
0
0 10 20 30 40 50 60 70 80 90 100
Timing of the cleavage (hpi)
first cleavage (t_1)
second cleavage (t_2)
third cleavage (t_3)

B


Percent cleaved
100
50
0
0 10 20 30 40 50 60 70 80
Duration of Δ_{12} (h)

C

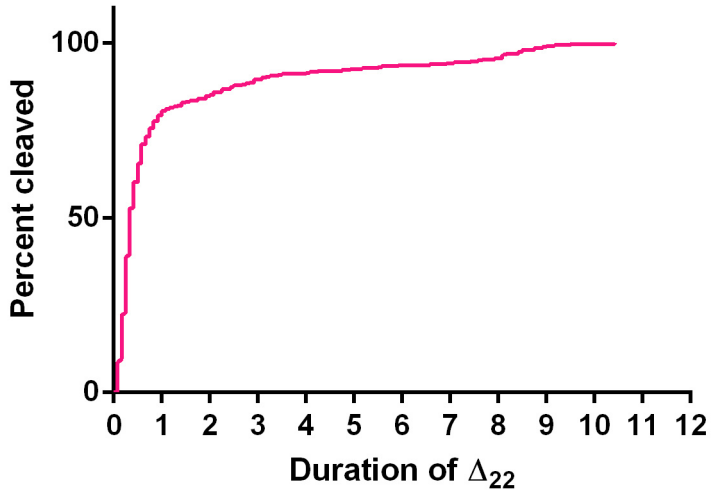
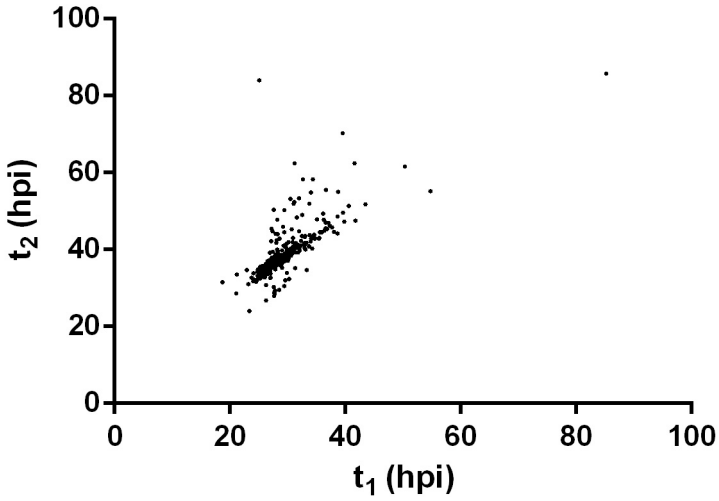

Percent cleaved
100
50
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Duration of Δ_{22}

Figure 7: Time curve of the first, second and third cleavage

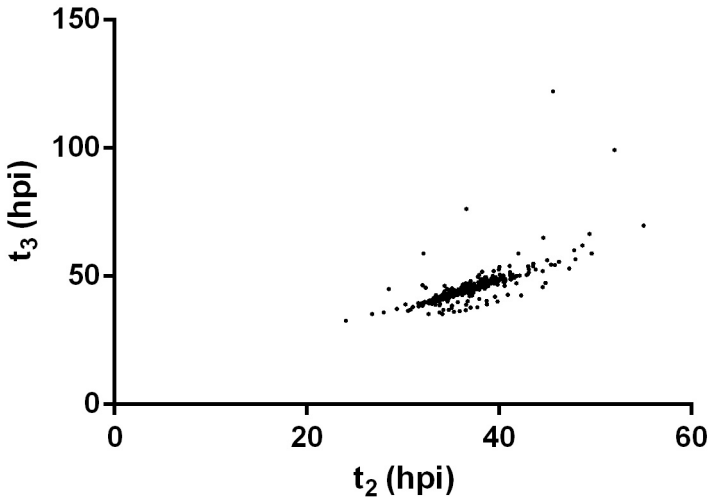
A: Timing of the first, second and third cleavage (t_1, t_2, t_3) of all embryos
B: Timing between the first and the second cleavage (Δ_{12})
C: Synchronicity of the second cleavage (Δ_{22})

The timing of the first three cleavage divisions showed a positive correlation among each other. Thus an early timing of the first cleavage in hpi strongly correlates with an early timing of the second cleavage ($r=0.75$, $p<0.001$) and also correlates with an early occurrence of the third cleavage ($r=0.62$, $p<0.001$). Furthermore, an early timing of the second cleavage in hpi was connected to an early timing of third cleavage ($r=0.71$, $p<0.001$).

A


100
80
60
40
20
0
t_2 (hpi)
0
20
40
60
80
100
t_1 (hpi)

B


150
100
50
0
t_3 (hpi)
0
20
40
60
t_2 (hpi)

C

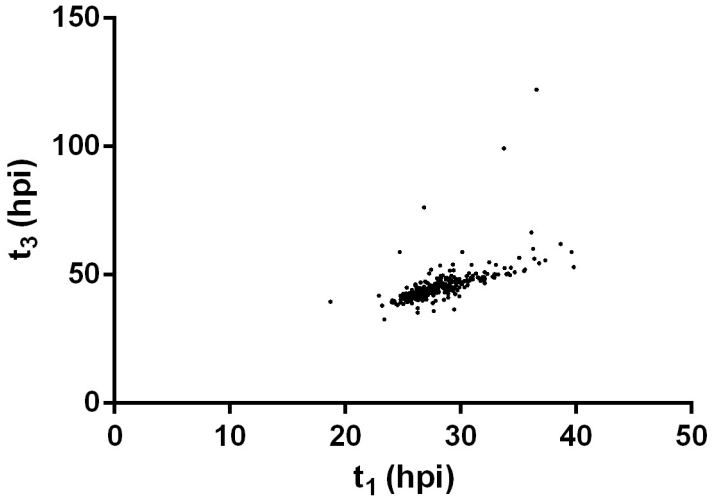

150
100
50
0
t_3 (hpi)
0
10
20
30
40
50
t_1 (hpi)

Figure 8: Correlation of t_1, t_2 and t_3

A: t_1 and t_2 show a high positive correlation (r=0.75, p<0.001)
B: t_2 and t_3 show a high positive correlation (r=0.62, p<0.001)
C: t_1 and t_3 show a high positive correlation (r=0.71, p<0.001)

In the following the timing of cleavage events after in vitro and in vivo maturation are compared. Mean values were compared using the Mann-Whitney-U test. The first, second and third cleavage appeared in a wide time window in both groups (Table 8). The time frame for the first cleavage after in vivo maturation was smaller, but within the time range of the first cleavage after in vitro maturation. The mean values of both time windows did not differ significantly (p=0.184). The second cleavage occurred between 24.1 and 85.8 hpi in general and there was no significant difference in timing between in vitro and in vivo matured embryos (p=0.133). The time range for the third cleavage was again very wide. Very late cleaving embryos occurred especially after in vitro maturation (35.3 hpi to 122 hpi). Accordingly, the in vivo matured oocytes run into the third cleavage around two hours earlier in average than in vitro matured ones (p=0.005). The value Δ_{12} was introduced meaning the time between the first and second cleavage, which is equivalent to the duration of the two cell stage. The mean Δ_{12} did not differ between both groups (p=0.405) and was observed to have a wide time range between 0.33 and 58.93 hpi. Illustrating the synchronicity of the first and second blastomere of the second cleavage, individual embryos showed a Δ_{22} of up to 10.4 hours. In comparison, the in vivo matured oocytes showed a significantly smaller Δ_{22} and thus cleaved more synchronous than the in vitro matured ones (p<0.001).

Table 8: Time window and mean value for t_1, t_{21}, Δ_{12}, Δ_{22} and t_3 after in vitro and in vivo maturation

		Minimum (hpi)	Maximum (hpi)	Mean ± SD (h)	P-value
t_1	*in vitro*	18.7	91.5	29.4 ± 5.8	0.184
	in vivo	23.4	72.2	30.1 ± 7.1	
t_{21}	*in vitro*	26.8	85.8	38.4 ± 6.4	0.133
	in vivo	24.1	70.3	38.1 ± 7.4	
t_3	*in vitro*	35.3	122.2	45.7 ± 7.8	0.005
	in vivo	32.6	60.2	43.8 ± 4.6	

Δ_{12}	*in vitro*	0.3	58.9	9.3 ± 4.4	0.405
	in vivo	0.5	30.8	9.3 ± 4.1	
Δ_{22}	*in vitro*	0.1	10.4	1.4 ± 2.3	<0.001
	in vivo	0.8	8.2	0.6 ± 1.2	

2.3. Abnormal morphology of the first cleavage

Beside normal cleavage, three abnormal cleavage patterns were observed during the first cytokinesis: the cleavage into two unequally sized blastomeres, the direct cleavage into three or four cells, and the cleaving into multiple fragments in the perivitelline space (Figure 9).

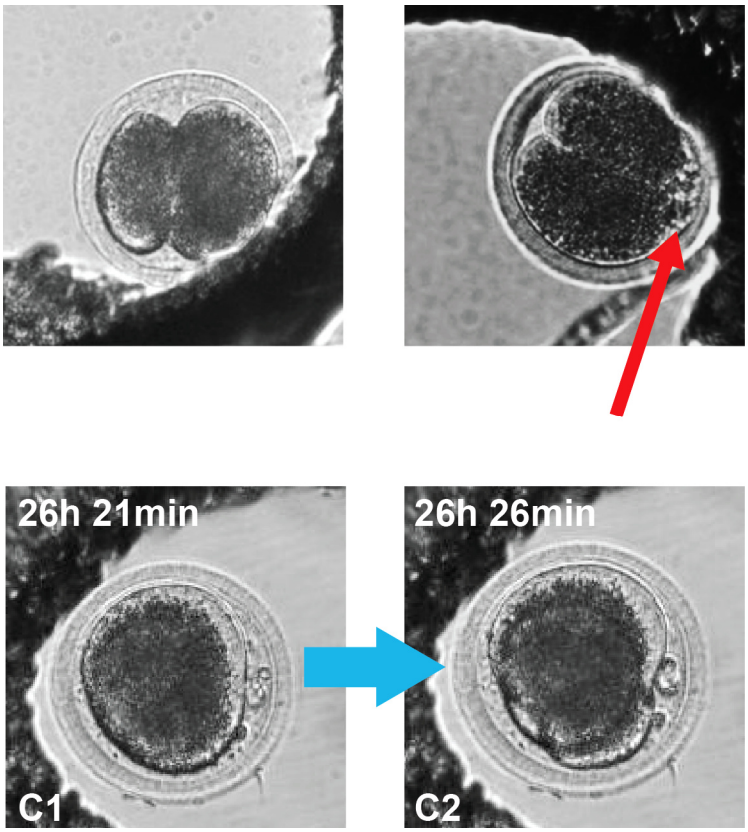


Figure 9: Abnormal first cleavage

A: Cleavage into two unequal sized blastomeres, B: Cleavage into two blastomeres with fragmentation (red arrow), C1 and C2: direct cleavage into the 3-cell stage within 5 minutes

Normal and abnormal cleavage morphology occurred after in vitro and in vivo maturation. 19.6% of the vitro matured oocytes showed an abnormal cleavage

pattern, compared to 16.3% of the in vivo matured oocytes. However, this difference was not significant (p=0.548). The most commonly observed abnormal cleavage pattern was the direct cleavage into the 3- or 4-cell stage with 12.1% in the in vitro matured group and 11.5% of the in vivo matured group. Unequal cleavage was observed in 6.3% of the in vitro group and in 3.9% of the in vivo group. Fragmentation only occurred as a portion of 1.1% and 1.0% after in vitro and respectively in vivo maturation.

2.4. Timing and development of the blastocyst stage at day 8

When in vitro and in vivo matured embryos were compared regarding their timing of later developmental stages, the advantage of the in vivo matured embryos was still significant. Thus the morula stage appeared earlier after in vivo maturation compared to in vitro maturation (119.1±9.4 hpi vs. 123.7±9.1 hpi, $p<0.001$), as did the start of blastulation (132.1±12.1 hpi vs. 137.8±15.3 hpi, p=0.012), and the reaching of the full blastocyst stage (149.0±14 hpi vs. 158.1±15.1 hpi, $p<0.001$). This developmental advance of the in vivo matured embryos continued until the blastocysts hatched, but was no longer significant at this stage (172.9±9.1 hpi vs. 175.8±8.1 hpi, p=0.087).

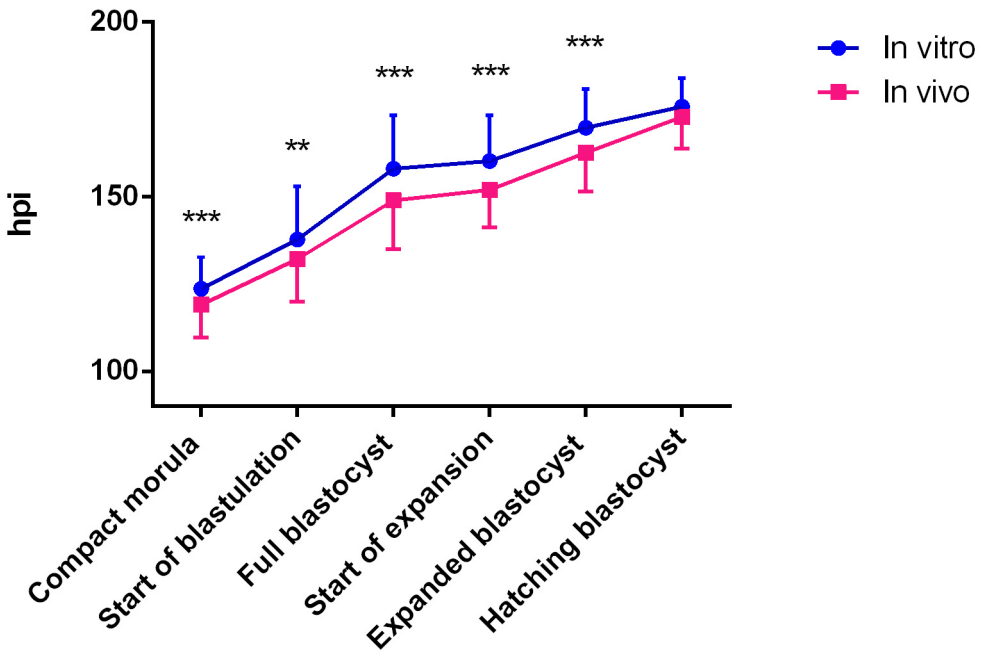


Figure 10: Development from the compact morula stage to the hatching blastocyst stage after in vitro and in vivo maturation

Embryos after in vivo maturation developed faster as they showed an earlier timing in all stages.

Data is shown as means ± SD, differences are tested with the Mann-Whitney-U test, *p=0.05, **p=0.001, ***p=0.001

These developmental differences could also be observed when the blastocysts were evaluated at 186 hpi. Accordingly, after in vivo maturation, 64.1% of the blastocysts were already in the hatching stage and 23.4% were at least expanded blastocysts. In the in vitro group only 31% were hatching and most embryos were either in the full (24%) or expanded blastocyst stage (32.5%).

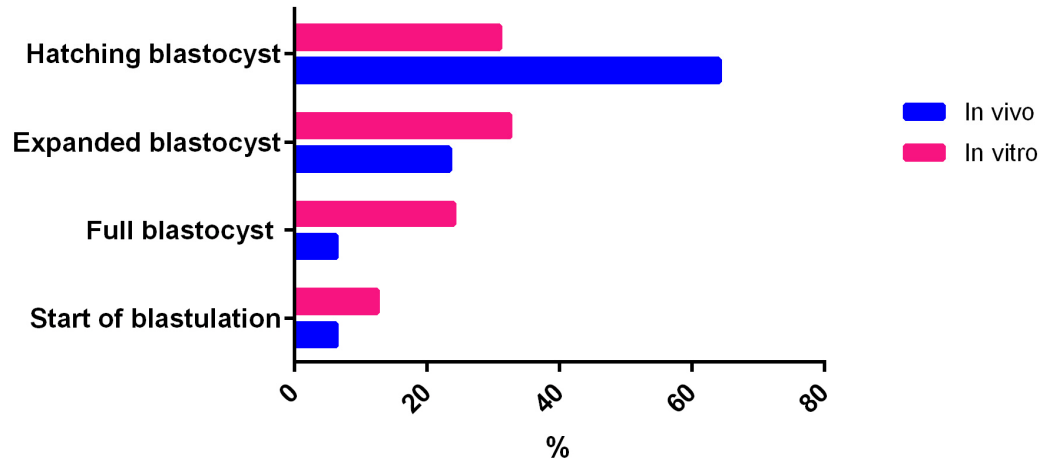


Figure 11: Blastocyst development at 186 hpi after in vivo and in vitro maturation

Embryos from in vivo matured oocytes are more advanced in their development and show a higher rate of hatching blastocysts.

During expansion and until hatching, some blastocysts showed a collapse of their blastocoel and a subsequent re-expansion. These collapses were documented in number for each embryo.

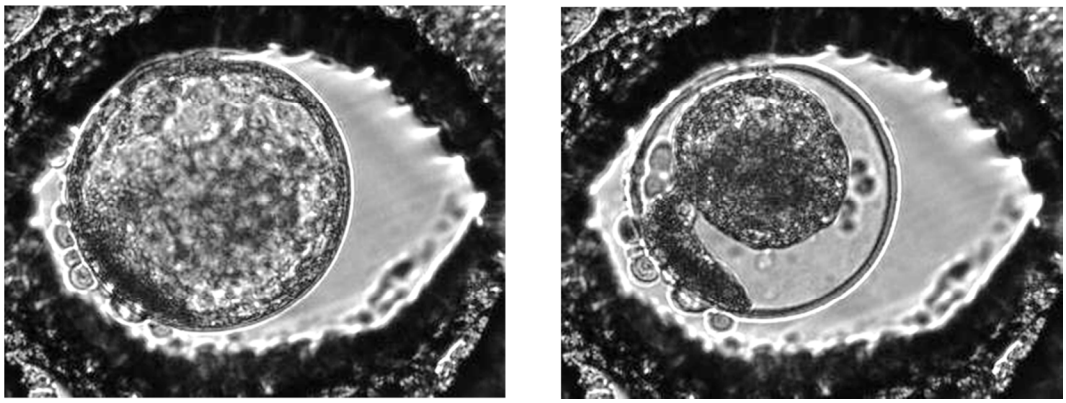


Figure 12: Contraction of the blastocoel

Expanded blastocyst (left side) and the same blastocyst after its blastocoel has collapsed. The expanded zona pellucida usually remains in the same size.

Embryos that hatched showed one or more collapses significantly more often than embryos that did not hatch (65% vs. 43%, p=0.001). Also the number of collapses did not seem to have a negative effect on the hatching rate (Figure 13). Blastocysts after in vitro maturation showed significantly less collapses of the blastocoel compared to in vivo matured ones (48% vs. 66%, p=0.014).

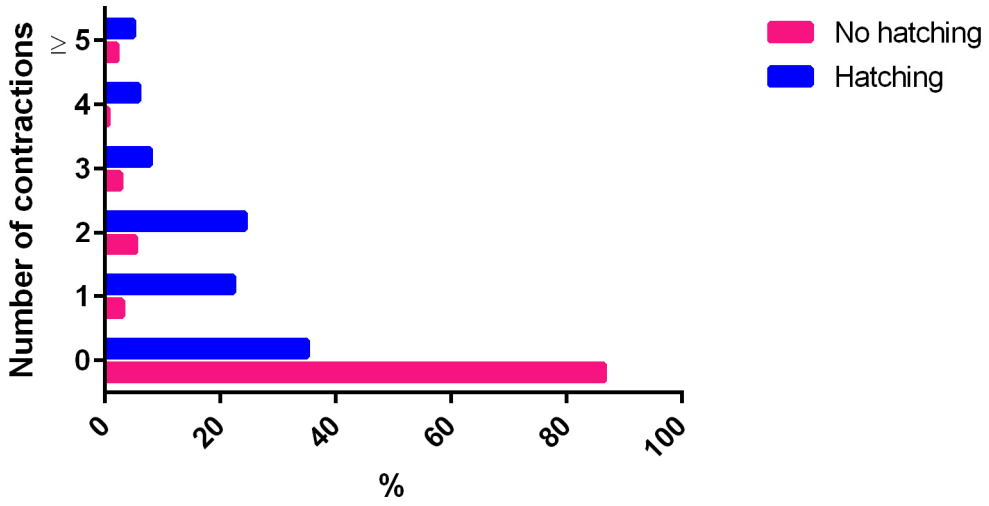


Figure 13: Blastocyst collapse until hatching

The collapse of the blastocoel did not have a negative effect on the hatching process of the blastocyst.

2.5. Prediction of the developmental competence based on early embryonic cleavage

2.5.1. Effect of timing of the first three cleavage divisions and the development to the blastocyst stage

In the following analyses, all embryos that were analyzable were included (n=541). The type of maturation was not regarded for this part of the analysis. Embryos that reached the blastocyst stage generally showed a smaller time range for the first, second and third cleavage compared to embryos that arrested during their development. Very late cleaving embryos only occurred in the group of arrested embryos. Most important was that embryos which reached the blastocyst stage were significantly faster in the average development regarding the timing of the first (27.7 hpi vs. 31.5 hpi , $p<0.001$), second (36.0 hpi vs. 41.1 hpi, $p<0,001$) and third (44.0 hpi vs. 47.8 hpi, $p<0.001$) cleavage compared to embryos that arrested in earlier stages (Figure 14). Furthermore the time interval between the first and the second cleavage (Δ_{12}), meaning the duration of the 2-cell stage, was significantly shorter for embryos with a high developmental competence (8.3 vs. 10.5, $p<0.001$). Accordingly, embryos that failed to reach the blastocyst stage showed significantly less synchronicity of the second cleavage and thus a high value for Δ_{22} (0.6 vs. 1.9, $p<0.001$).

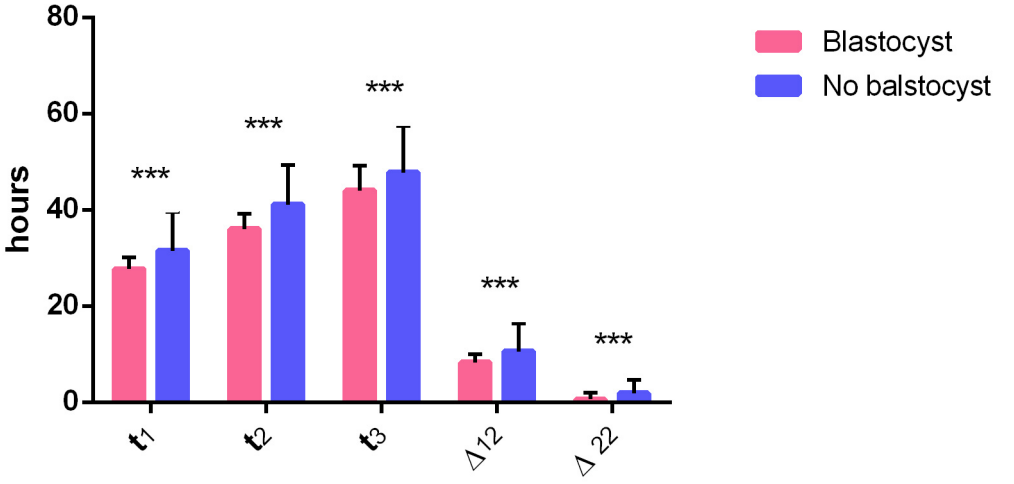
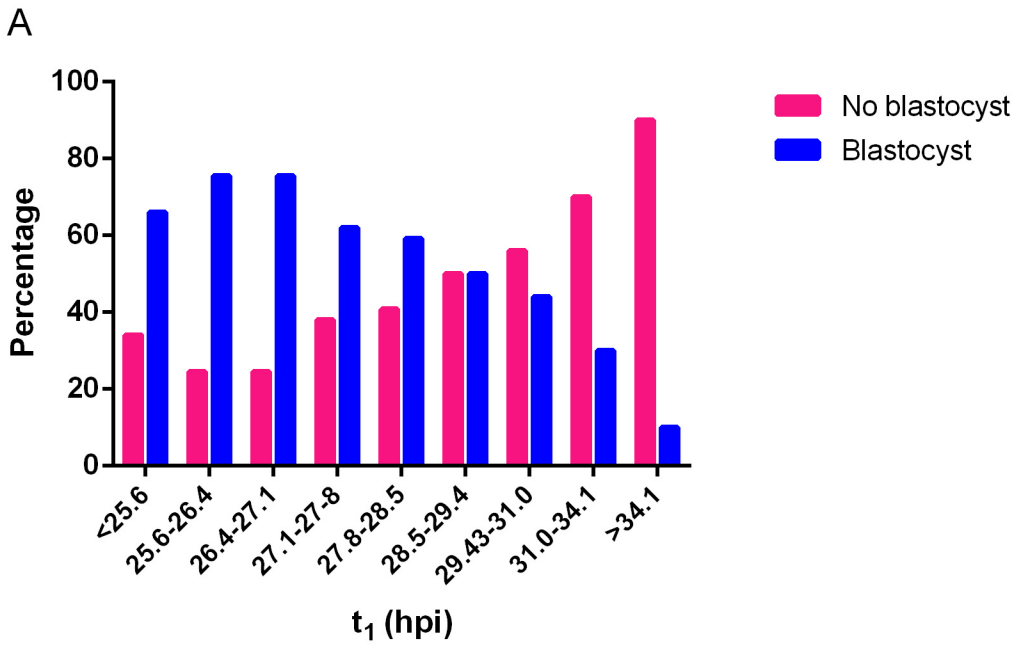


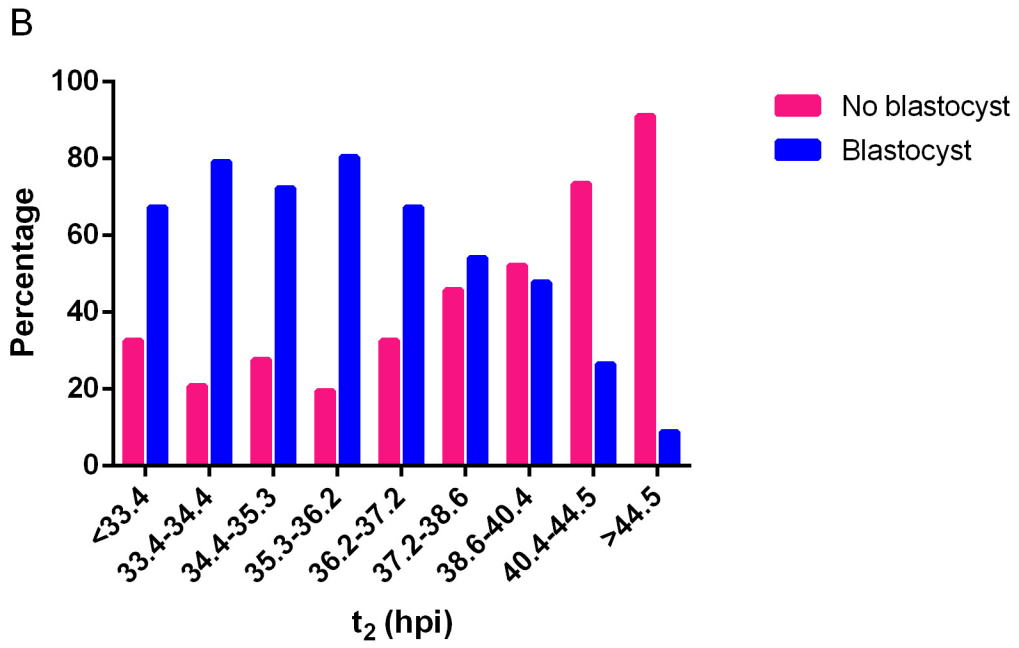
Figure 14: Timing of t_1, t_2, t_3, Δ_{12} and Δ_{22} of embryos that reached the blastocyst stage and arrested ones

Data are shown as means ± SD, significant differences were tested with the Mann-Whitney-U-test, *p=0.05, **p=0.001, ***p<0.001

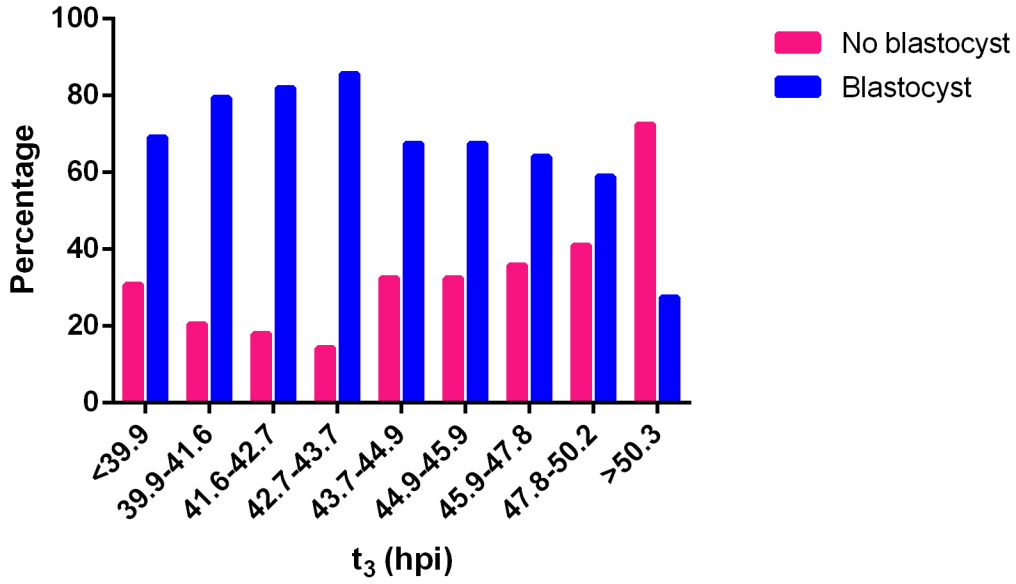
In the following, the data was analyzed to find out whether the cleavage times had an effect regarding the reaching of the blastocyst stage. As shown in chapter 2.2, the number of embryos cleaving at different time points is very variable. To be able to compare the blastocyst rate at a given time of cleavage, percentiles of the timing with an equal number of embryos were determined and compared. Accordingly, a first cleavage until 28.5 hpi resulted in a blastocyst rate higher than 50%. The highest blastocyst rate (~75%) was reached, when the first cleavage occurred between 25.6 and 27.1 hours. Very early (before 25.6 hpi) and very late cleaving embryos (later than 28.5 hours) showed a decreased blastocyst rate. The later the first cleavage, the lower the blastocyst rate, until this correlation reached the lowest a rate of only 10% at a first cleavage around 34.1 hpi. An early beginning of the second cleavage (until 38.6 hpi) also resulted in a higher blastocyst rate of more than 50%, which again demonstrates the correlation between the first and the second cleavage. The optimal time range for the second cleavage seemed to be between 33.4 and 36.2 hpi, as these percentiles showed the highest blastocyst rate. Embryos with a late second cleavage showed a blastocyst rate of less than 50%. Regarding the timing of the third cleavage, the blastocyst rate increased up to 86% when t_3 was lower than 43.7 hpi. If the third cleavage began after 43.7 hours, the blastocyst rate decreased with every percentile. It has

to be mentioned that a very early timing of the first, second, or third cleavage also seems to have a negative effect on the blastocyst rate. The optimal duration of the two cell stage until the beginning of the second cleavage (Δ_{12}) appeared to be between 7.7 and 8.6 hours with a blastocyst rate between 77% and 81%. If Δ_{12} was too small or too large, the blastocyst rate dropped. Regarding Δ_{22}, meaning the synchronicity of the second cleavage, the first two percentiles, (cleaving within 0.25 hours) resulted in the highest blastocyst rates (75%-77%). Lower synchronicity of the second cleavage had a negative effect on the blastocyst rate.

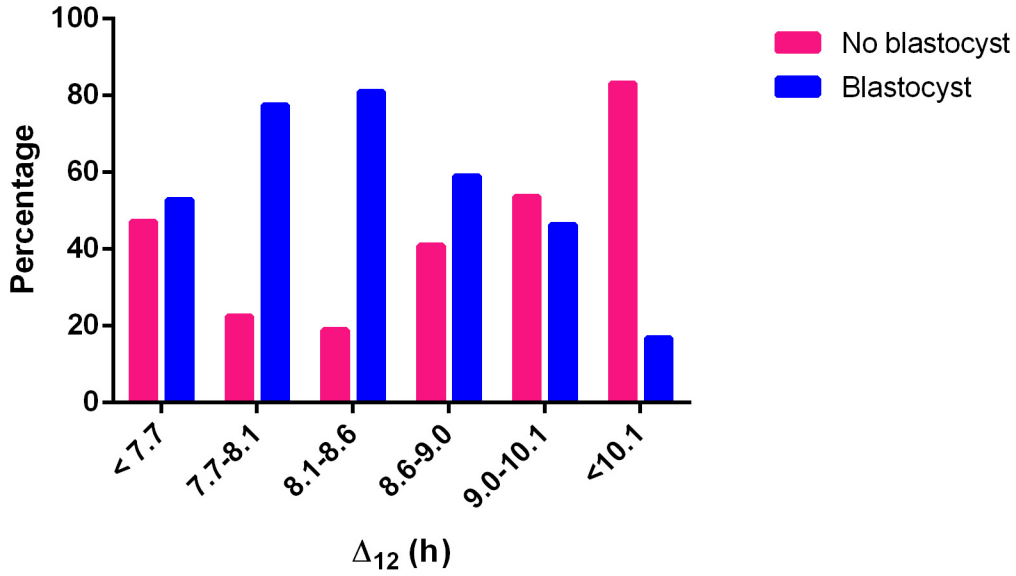

A
100
80
60
40
20
0
Percentage
No blastocyst
Blastocyst
<25.6
25.6-26.4
26.4-27.1
27.1-27-8
27.8-28.5
28.5-29.4
29.43-31.0
31.0-34.1
>34.1
t1 (hpi)


B
100
80
60
40
20
0
Percentage
No blastocyst
Blastocyst
<33.4
33.4-34.4
34.4-35.3
35.3-36.2
36.2-37.2
37.2-38.6
38.6-40.4
40.4-44.5
>44.5
t2 (hpi)

C


100
80
60
40
20
0
Percentage
No blastocyst
Blastocyst
<39.9
39.9-41.6
41.6-42.7
42.7-43.7
43.7-44.9
44.9-45.9
45.9-47.8
47.8-50.2
>50.3
t3 (hpi)

D


100
80
60
40
20
0
Percentage
No blastocyst
Blastocyst
<7.7
7.7-8.1
8.1-8.6
8.6-9.0
9.0-10.1
<10.1
Δ12 (h)

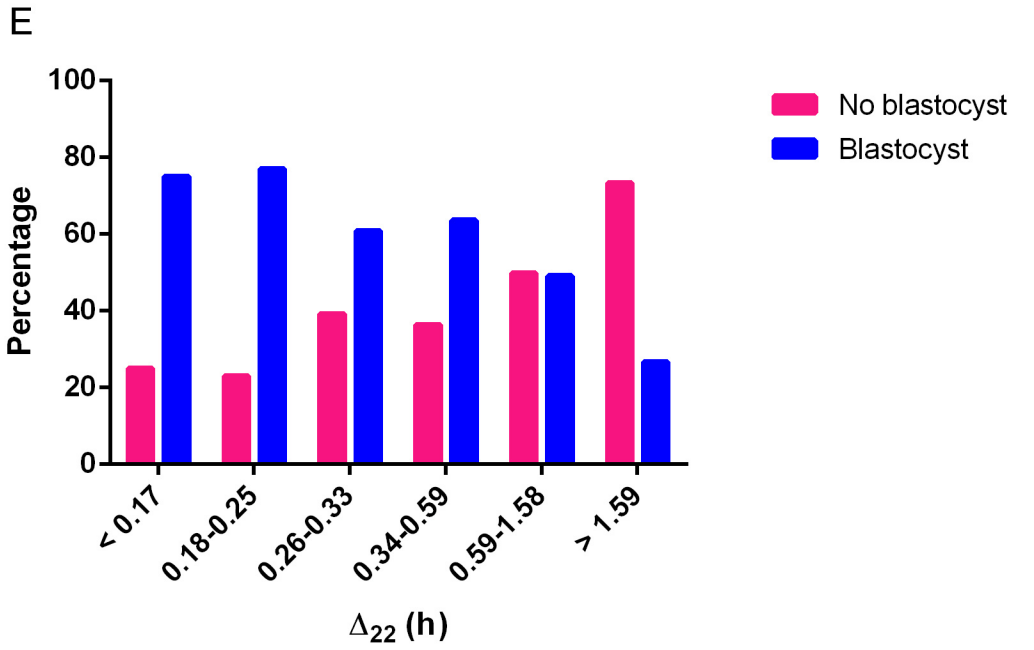


Figure 15: Blastocyst rate at different time intervals of t_1, t_2, t_3, Δ_{12}, Δ_{22}

Time intervals were divided based on percentiles, accordingly every time interval includes an equal number of embryos

A: Early timing of the first cleavage had a positive effect on the blastocyst rate *(optimal time range: 25.6-27.1 hpi)*

B: Early timing of the second cleavage had a positive effect on the blastocyst rate *(optimal time range: 33.4-36.2 hpi)*

C: Early timing of the third cleavage had a positive effect on the blastocyst rate *(optimal time range: 34.0-43.7 hpi)*

D: Very short and very long time between the first and second cleavage had a negative effect on the blastocyst rate. *(optimal time range: 7.7-8.6 h)*

E: Less synchronicity of the second cleavage had a negative effect on the blastocyst stage *(optimal time range: < 0.25 h)*

2.6. Effect of the morphology of the first cleavage on the development to the blastocyst stage

The developmental competence to the blastocyst stage after normal cleavage and the different abnormal cleavage patterns are shown in Table 9. After an abnormal first cleavage, the blastocyst rate of the cleaved zygotes was in general reduced significantly from 56% to 31.4% ($p<0.0001$). Beside direct cleavage into 3-/4-cell stage (blastocyst rate=37.0%) and cleavage with fragmentation (blastocyst rate=40.0%), cleavage into two unequally sized blastomeres seems to have the

most negative effect on further embryo development, since the blastocyst rate declined to 26.9%.

	Normal cleavage	Unequal cleavage	Direct 3-/4-cell stage	Fragmented cleavage
Blastocyst rate*	56.0%	26.9%	37.0%	40.0%

Table 9: Blastocyst rate after normal and abnormal morphology of the first cleavage

*The percentages present the rate of blastocysts out of the embryos that cleaved. In vitro and in vivo matured embryos are not listed separately since there was no significant difference in the frequency of abnormal cleavage between both groups.

2.6.1. Logistic regression model: prediction of the development to the blastocyst stage

Two logistic regression models were selected by Dr. Christiane Fuchs from the Institute for Bioinformatics and Systems Biology at the Helmholtz Zentrum Munich to predict the chance of an embryo to reach the blastocyst stage. The first model (model A) includes the co-variables t_1^2, Δ_{12}, the morphology of the first cleavage and the type of maturation. t_1^2 was included into the regression model, since there seemed to be a quadratic effect of the timing of the first cleavage on the blastocyst outcome. The second model (model B) predicts the blastocyst rate based on the varibales t_1^2, Δ_{12} and $\Delta_{22.}$ Table 10 and Table 11 show the results for both models.

Table 10: Logistic regression Model A

Variables	**β[a]**	**P-value[b]**	**Odds ratio**
$\mathbf{t_1^2}$	-0.003	<0.001	0.997
$\mathbf{\Delta_{12}}$	-0.25	<0.001	0.779
$\mathbf{Morph_{unequal}}$	-1.349	0.02	0.260

$Morph_{direct\ 3/4}$	-0.627	0.08	0.534
$Morph_{fragmented}$	0.676	0.62	1.966
Maturation	0.454	0.12	1.575
Intercept	5.290	<0.001	198.339

Table 11: Logistic regression Model B

Variables	**β[a]**	**P-value[b]**	**Odds ratio**
t_1^2	-0.004	<0.001	0.996
Δ_{12}	-0.266	<0.001	0.767
Δ_{22}	-0.396	<0.001	0.673
Intercept	6.245	<0.001	515.556

[a] Coefficient estimate of logistic regression
[b] P value of Wald chi-squared statistic

The estimated β-coefficients were inserted into the logistic regression function in order to predict the probability to reach the blastocyst stage:

Model A:

$$z = 5.29 - 0.003\ (t_1^2) - 0.25(\Delta_{12}) - 1.349\ (Morph_{unequal}) - 0.627\ (Morph_{direct\ ¾}) + 0.676\ (Morph_{fragmented}) + 0.454\ (Maturation)$$

Model B:

$$z = 6.245 - 0.004\ t_1^2 - 0.266\ \Delta_{12} - 0.396\ \Delta_{22}$$

Model B confirms that the probability for an embryo to the blastocyst stage decreases with: 1) delayed timing of the first cleavage, 2) a prolonged duration of the 2-cell stage (Δ_{12}) and 3) a prolonged duration of the second cleavage (Δ_{22}) ($p<0.001$). Model A shows that the occurrence of an unequal cleavage reduces the chance to reach the blastocyst stage about 74% ($p=0.02$). Other morphology

patterns of the first cleavage and the type of maturation did not have a significant effect.

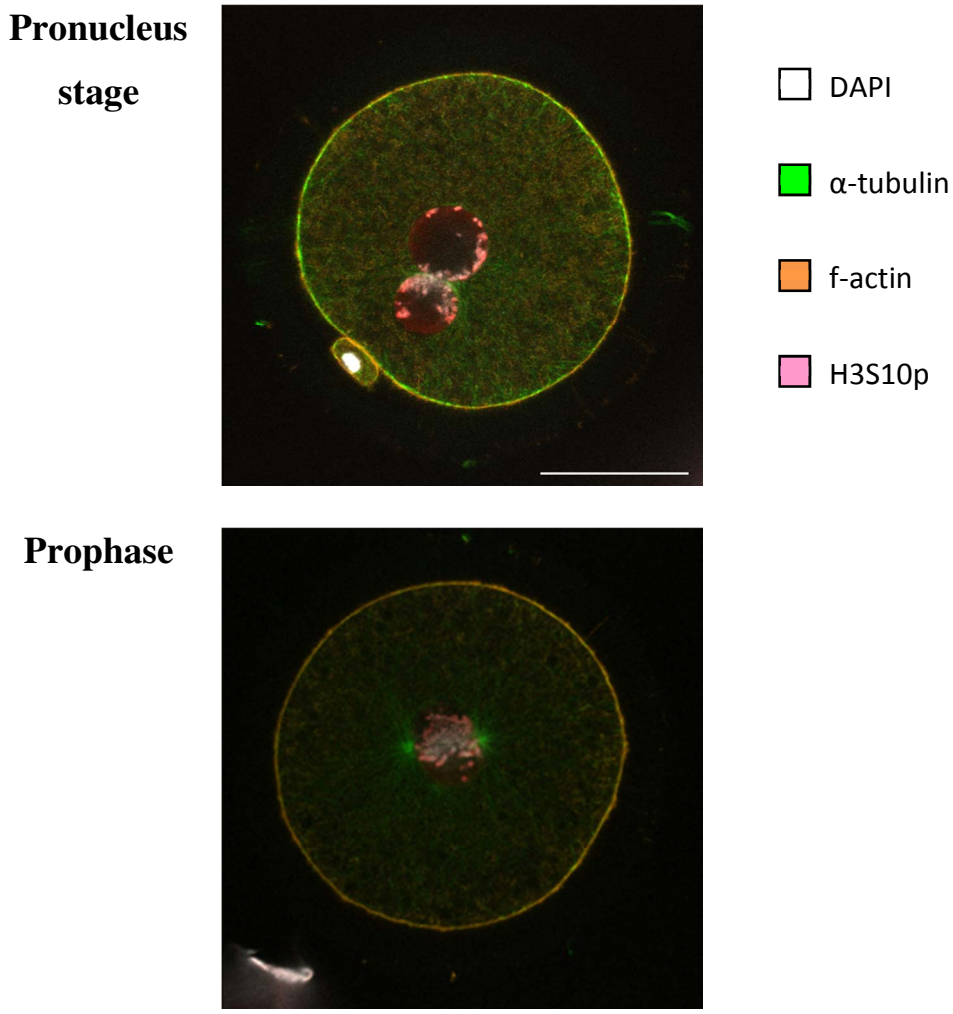
To calculate the predictive probability for an embryo regarding reaching of the blastocyst stage, the values for the time points or durations in hpi have to be inserted into the functions. The accuracy of prediction for both models was tested by cross validation. Accordingly, the overall rate of correctly classified outcome was 72% for model A and 76% for model B. With model A, the sensitivity was 86% and the specificity was 44%. For model B the sensitivity was 93% and the specificity was 47%.

3. CLSM analyses

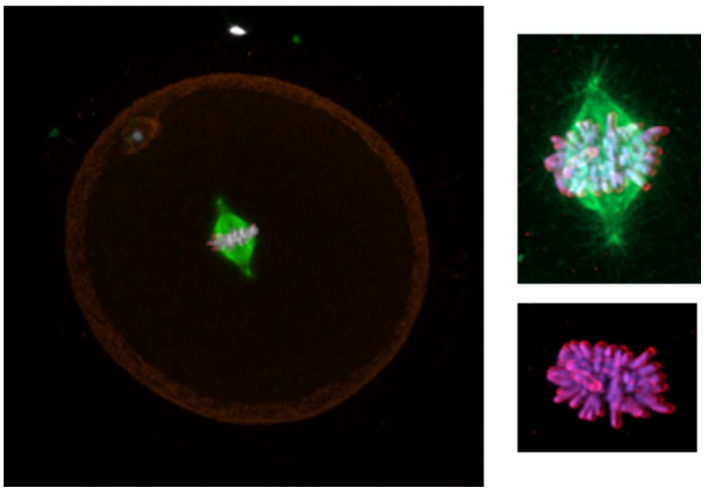
3.1. Reference data for normal first mitotic cleavage division

In total 382 embryos were stained after fixation and following analyzed with a confocal microscope by Dr. Felix Habermann. This data served as reference for getting an overview over the morphology of the first mitotic cell cleavage in bovine embryos. The attention was primarily focused on the geometry and the structure of the mitotic spindle apparatus and the chromosomes during mitosis. Out of the 382 embryos, 95% (n=375) were fertilized with at least one sperm. The images show snapshots of embryos in different stages of mitosis including the pronucleus stage, the prometaphase, the metaphase, the anaphase, the telophase and the cytokinesis into two blastomeres (Figure 16). The image panels present single optical sections and maximum intensity projection (MIP) renderings of confocal image stacks. DNA (stained with DAPI) is pseudo-colored in white or blue, H3S10p is shown in red/magenta. Microtubules (MTs) are shown in green and F-actin (stained with phalloidin) in orange/brown. Embryos that were fixated in the pronucleus stage showed two pronuclei, a female and a male one. During early pronucleus stage, the two pronuclei appeared widely spaced, while they approached each other with advancing development. Embryos in the prophase showed condensed chromosomes, and the mitotic spindle began to assemble around the two centrosomes. During the metaphase stage, the chromosomes are aligned in the equatorial layer and the centrosomes are located at the poles of the spindles. Snapshots of the anaphase showed the poles in different positions as they are drifting apart into the opposite direction and simultaneously the sister chromatids get separated as they move toward the spindle poles. During telophase

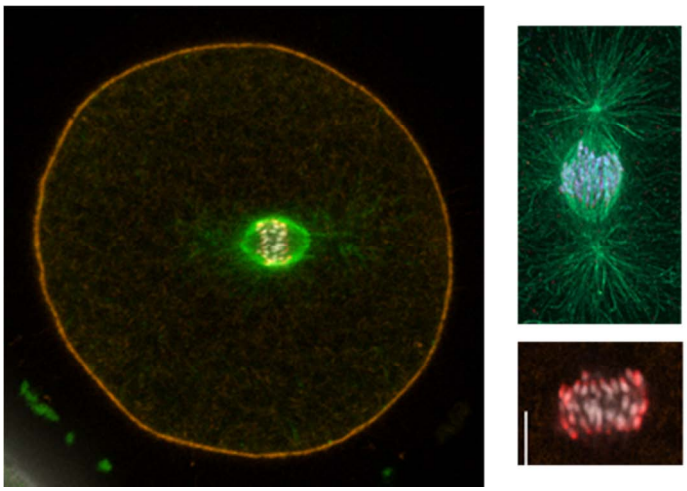
the chromosomes further moved until they arrive at the centrosomes. Images of the telophase show the division of the cytoplasm at different stages ending with the complete division into two separate blastomeres. A midbody consisting of remaining microtubuli is was often visible during telophase and after cytokinesis (Figure 16).



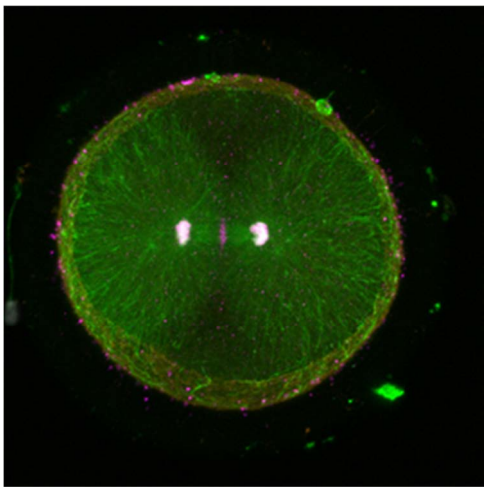
Metaphase



Anaphase



Telophase



Cytokinesis

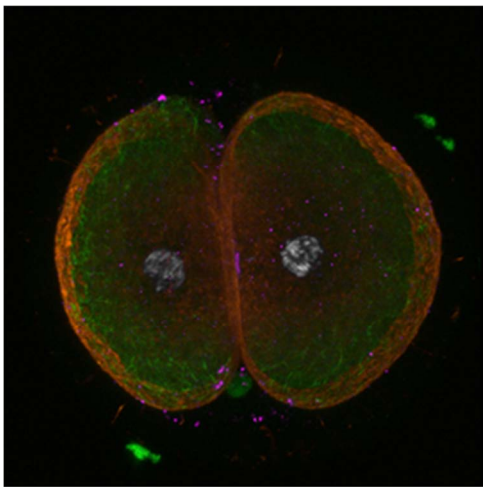


Figure 16: Stages of the first cleavage

A) Pronucleus stage: The male and the female pronucleus have already approached each other (single optical section, scale bar = 50 µm).
B) Prophase: The mitotic spindle starts to assemble around the condensed chromosomes (single optical section).
C) Metaphase: The condensed chromosomes are aligned in the equatorial layer in the mitotic spindle. (MIP renderings of a central large-scale image stack and of a high resolution stack of the metaphase spindle, scale bar = 10 µm)
D) Anaphase: The centrosomes have moved further apart from each other and are connected to the spindle aster by long compact bunches of microtubules, the sister chromatids are already separated (Single optical section and MIP rendering of the spindle and the astral microtubules, scale bar = 10 µm).
E) Telophase: The chromosomes have moved towards the spindle poles. The immunofluorescence staining for H3S10p delineates the area of the midbody (MIP renderings of central image stacks).
F) Cytokinesis: The two separated blastomeres contain the nuclei with the decondensed chromosomes. A midbody is again delineated (MIP renderings of central image stacks).

(Courtesy of Dr. Felix Habermann)

The described stages were observed more or less frequently depending on the timing of fixation. In the group that was fixated at 22 hpi, the only stage that could be observed was the pronucleus stage. Around 24 hpi a small percentage of the embryos was in the metaphase stage (3%) or already running through the anaphase, telophase or cytokinesis (3%). Two hours later the number of embryos in the pronucleus stage was still high (76%) but the percentage of further stages especially the metaphase stage, were larger and the first 2-cell stage embryos appeared. From 28 hpi on, the number of embryos in the 2-cell stage increased, while prior stages appeared less often. At 30 and 32 hpi the 2-cell stage was the

most frequently observed cell stage (67%), while the other stages appeared with declining frequency.

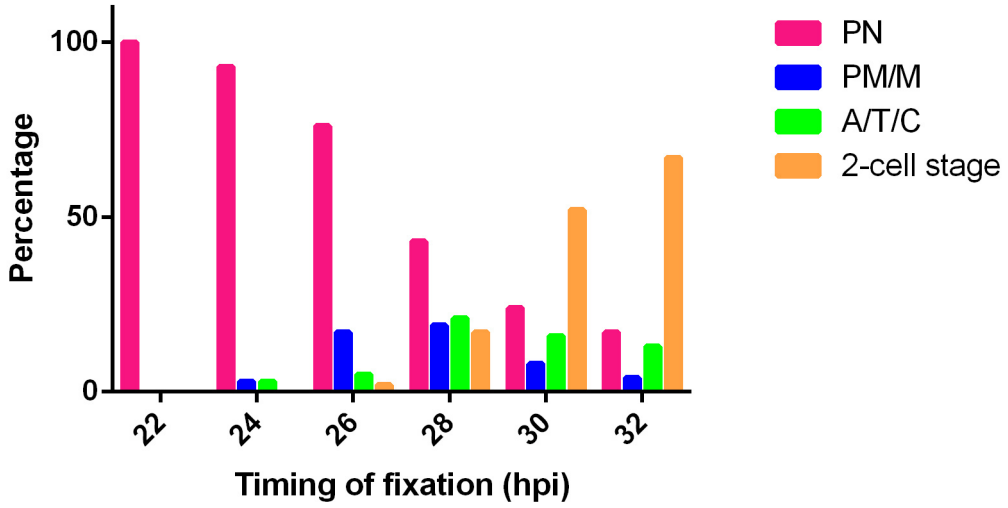


Figure 17: Stages of the first cleavage depending on the time post insemination

Following stages were observed with differing frequency: PN=pronucleus stage, PM/M= prometaphase/metaphase, A/T/C= anaphase/telophase/cytokinesis, 2-cell stage

It has to be kept in mind, that for the time-lapse imaging and for the CLSM analysis the embryos were cultured in different dishes and in different sizes of groups (time-lapse imaging: culture in 16-WOW dishes; fixation for CLSM analyses: culture in 4-well dishes in groups of 40 embryos) but with the same amount of culture medium per embryo. To check whether the embryos from the two culture systems are comparable regarding the timing of the first cleavage, the percentage of the 2-cell stage in the different time groups for the CLSM analyses was compared to the percentage of embryos that already began their first cleavage in the camera system until the corresponding time points. It is important to notice that for the fixated embryos the number of two cell stages is counted while in the live observation system the beginning of the cleavage into the two cell stage is documented. Since the cytokinesis might take some time, the timing for the occurrence of the definite 2-cell stage in the camera system is actually later than shown in Figure 18. Unfortunately the completion of the first cytokinesis cannot be evaluated in the camera system due to the high lipid accumulation in the bovine cytoplasm. Therefore it can be said with reservations that embryos cultured and observed in the live monitoring system appeared to be slightly earlier

in their development to the 2-cell stage, since most of the embryos under live observation cleaved between 26 and 28 hpi, in comparison to the fixated embryo where the highest increase of the percentage of two cell stage was between 28 and 30 hpi. At 32 hpi the percentage of the embryos in two cell stage was almost the same (67% after fixation vs. 69% under live observation). Considering that the measured time points for the 2-cell stage are actually not defined exactly the same, the difference in timing between both groups might not be as severe as shown in Figure 18, but yet a slight difference in the timing of the first cleavage due to different culture conditions in this experiment cannot be excluded completely.

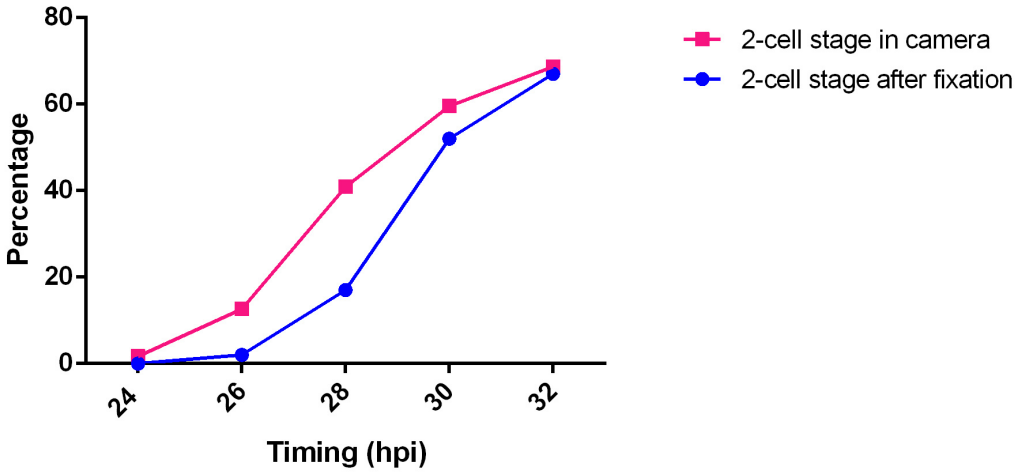


Figure 18: Timing of the occurrence of the 2-cell stage after fixation and under live monitoring conditions

The cumulative percentage of the embryos in the two cell stage are shown for 24, 26, 28, 30 and 32 hpi for embryos after fixation (violet) and embryos cultured in the camera system (blue). Embryos cultured and fixated for the CLSM analyses seem to be slightly retarded, which might be a result of the different ways to measure the timing of the two cell stage or caused by different culture conditions.

3.2. Polyspermic fertilization as a reason for abnormal first mitotic cleavage divisions

The images of normal appearing cleavage stages were compared to images with noticeable abnormalities in the mitotic cleavage. Several abnormalities regarding abnormal chromosome segregation or assembly of the mitotic spindle apparatus were observed. One main cause for abnormal first cell cleavage seemed to be

polyspermic fertilization. Accordingly, 19% of all analyzed embryos were fertilized with 2 sperms and 7% were fertilized with more than two sperms.

Table 12: Number of embryos with normal and polyspermic fertilization

Experiment	N (n)	UFO (n)	Normally fertilized (n)	Polyspermy (n)	2 sperms (n)	> 2 sperms (n)
1	181	12	126	43	37	6
2	201	11	133	54	34	20
Total	382	23	259	97	71	26

As the images are only snapshots of what is happening in an embryo after fertilization with two or more sperms, it can only be assumed how the different cleavage patterns emerge and how these embryos could either develop further or arrest. The most frequent abnormal patterns of the different mitotic stages and their most probable causes are shown schematically in Figure 19. The picture includes only some of the frequently observed cleavage patterns. Other combinations are possible. Additionally, there are embryos that are not exactly in one of the stages that are shown, but most of them can be matched to one cleavage path.

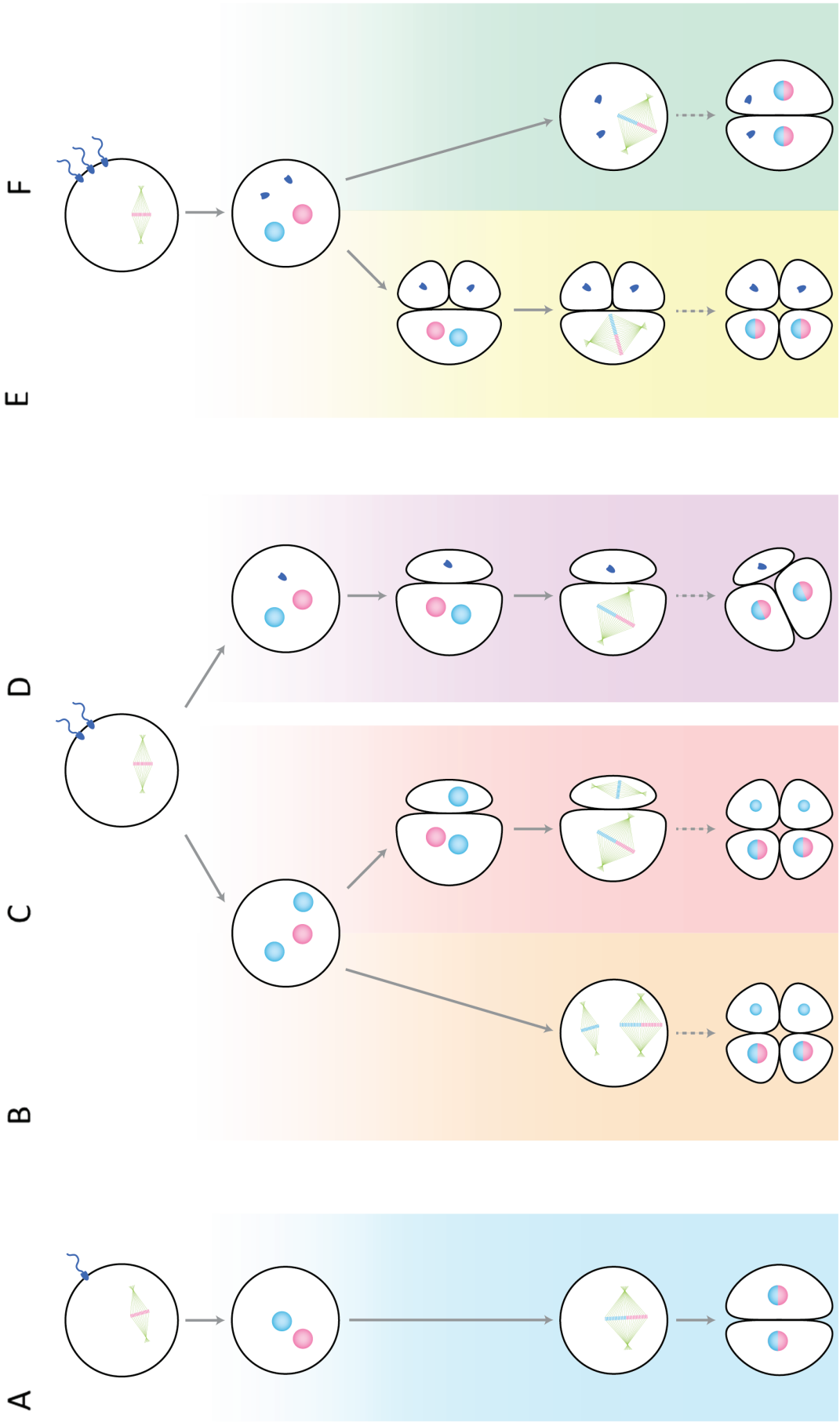


Figure 19: Normal and abnormal cleavage patterns observed in the CLSM analyses

The female DNA is shown in pink, the male DNA in blue. Male and female DNA cannot be distinguished in the images from the confocal microscopy, but the origin of the DNA can be assumed. For better understanding they are colored in the diagram.

Row A (blue) shows a normal first mitotic embryonic cleavage into the two cell stage. After the fertilization with one single sperm, two pronuclei develope, the chromosomes are separated by the mitotic spindle apparatus and the cytokinesis results into two blastomeres with one cell nucleus each.

Row B to D (orange, red and violet) show a zygote that is fertilized with two sperms. Usually one sperm forms a normal pronucleus. The additional sperm can either develop into a second male pronucleus (orange and red row) or appear as a non decondensed sperm head somewhere in the cytoplasm (violet row). Three pronuclei could result in two mitotic spindles, while one contains a diploid set of chromosomes and the other one contains only the haploid chromosome complement of the additional male pronucleus (orange row). The haploid metaphase spindle appears slightly smaller than the diploid one. After the separation of the sister chromatids, cytokinesis can result in an embryo with four blastomeres. Two blastomeres correspond to the blastomeres of a normal two cell stage with one nucleus each, consisting of maternal and the paternal DNA. The other two blastomeres contain nuclei with paternal DNA only.

The fertilization with two sperms can in some cases initiate a pseudo-cleavage into a 2-cell stage with one blastomere containing the additional pronucleus or the sperm head (row C and D, red and violet). We called this pseudo-cleavage, as a cytokinesis occurred, but chromosomes are still in the pronucleus stage. This pseudo-cleavage can result in a 2-cell stage with two equally sized blastomeres (not shown in the illustration) or into two unequally sized blastomeres. As illustrated in the red row, the male pronucleus can form a mitotic spindle with a haploid chromosome set, and accordingly the mitotic cleavage and cytokinesis results into a 4-cell stage with the same blastomere pattern as in the orange row. In case of a pseudo-cleavage caused by a non-decondensed sperm head (violet row), the blastomere with the two pronuclei can run through a normal cleavage, resulting in a 3-cell stage with two normal blastomeres containing a nucleus each and an additional blastomere containing the sperm head. A development to the blastocyst stage of this pseudo-3-cell stage embryo is certainly possible through

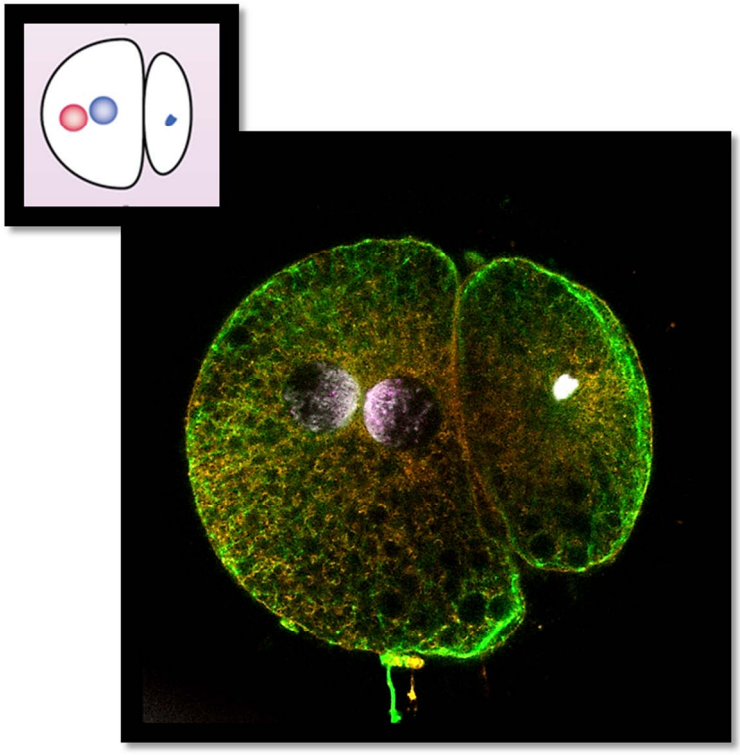
the arrest of the additional blastomere and further development of the two regular blastomeres containing the nuclei.

The case of fertilization with more than two sperms is shown in row E and F. It appeared that two male pronuclei were formed and the third sperm was found again being not decondensed somewhere in the cytoplasm. These additional pronucleus and the sperm head can again initiate a pseudo-cleavage and accordingly result in a pseudo-3-cell stage. In row E the three cell stage consist of one blastomere containing a sperm head, one blastomere containing a single male pronucleus and one blastomere with the actual male and female pronuclei. Due to two additional pronuclei in row F, the resulting 3-cell stage has two blastomeres with only a male pronucleus. In the normal blastomeres with a male and a female pronucleus, a diploid mitotic spindle can form. In blastomeres containing only a male nucleus a haploid mitotic spindle can occur. Accordingly the first mitotic cleavage results into a 5-cell stage in row E and in a 6-cell stage in row F.

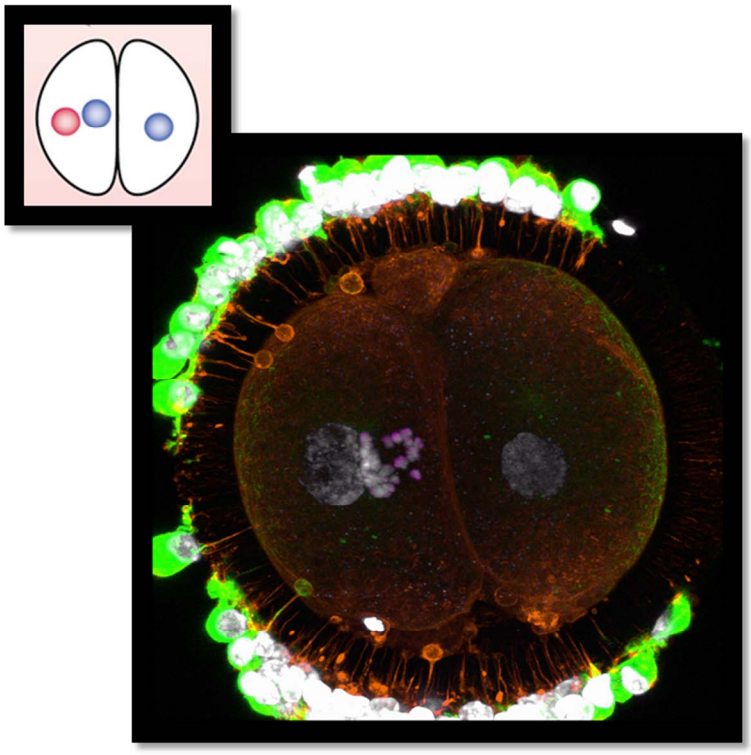
In principle it is also possible that one or more additional sperm heads are just lying in the cytoplasm and do not influence the first mitotic cleavage (not shown in the illustration).

Theoretically, a pseudo-cleavage that is initiated by an additional pronucleus or a sperm head can also occur at the same time as the normal cell cleavage and thus might often appear as a direct cleavage into a pseudo-3- or pseudo-4-cell stage.

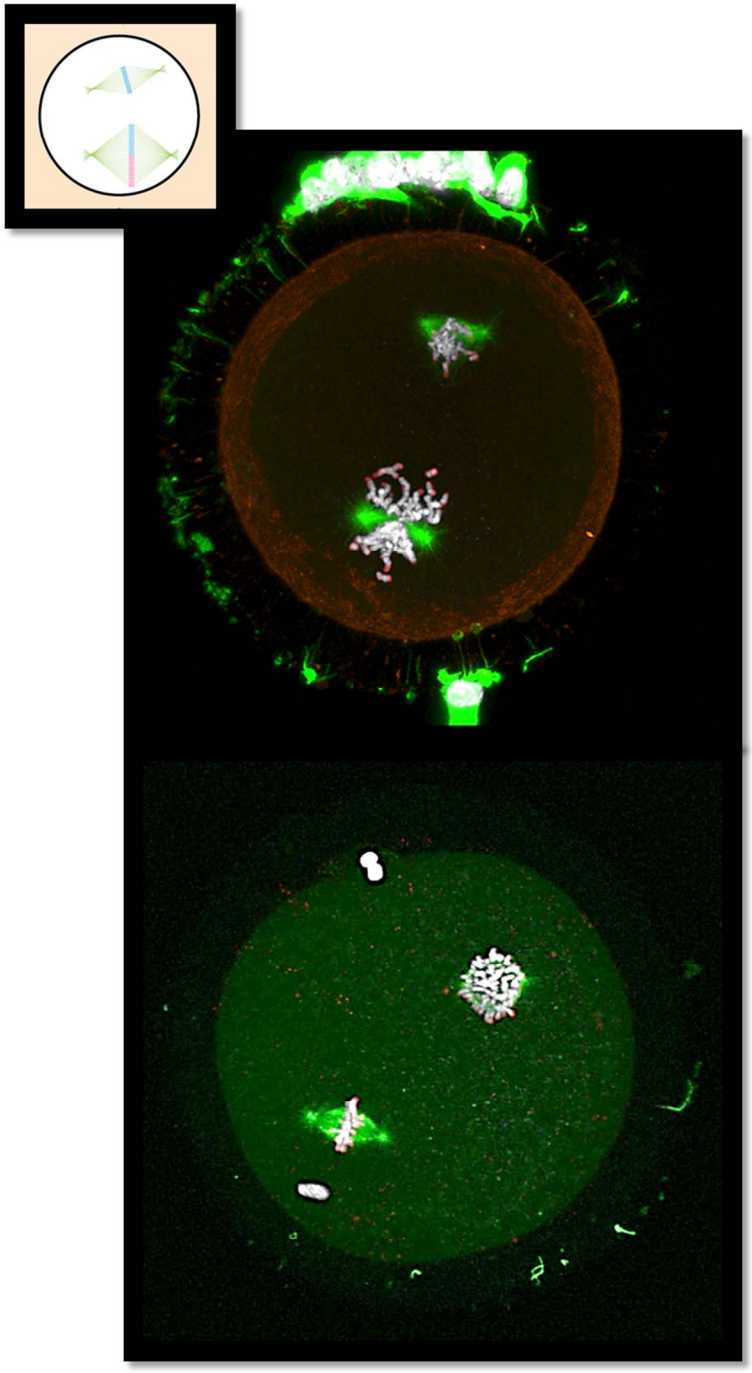
Examples for CLSM of embryos that were fertilized with one or more sperms are shown in Figure 20.



Dispermic fertilization: asymmetric pseudo-cleavage at the pronucleus stage, caused by an additional non-decondensed sperm head.



Dispermic fertilization: symmetric pseudo-cleavage at the pronuclear stage.



Dispermic fertilization: Two embryos in the metaphase; while one spindle is containing a normal diploid chromosome set, the second mitotic spindle only contains the haploid chromosome set of the additional sperm and appears often smaller.

Figure 20: 3-D images of embryos in the first mitotic cleavage division after polyspermic fertilization

(Courtesy of Dr. Felix Habermann)

Some of these polyspermic mitotic cleavage patterns can be associated with abnormal morphology of the first cleavage division which could be observed in

the time-lapse imaging. Accordingly, cleavage into unequally sized blastomeres and direct cleavage into the 3- or 4-cell stage can be explained by some cleavage morphologies observed in the 3D-images. On the other hand, embryos that appeared normal under the light microscope might actually be the result of a pseudo-cleavage and thus might actually not be as progressed as it seems. Since embryos with an abnormal first cleavage could also develop to the blastocyst stage, the additional blastomeres probably arrest and the blastomeres with a correct chromosome set would develop further.

V. DISCUSSION

1. Oocyte quality and further development after in vitro and in vivo maturation

To test the suitability of the WOW-dishes for the culture of bovine embryos, a control group of 16 embryos was cultured in the same amount of medium like in the camera system. Accordingly the blastocyst rate was significantly higher when the embryos were cultured under in the WOW-dishes compared to the control group (44% vs. 35%, p=0.01). This benefit of the WOW-culture system was already observed by Vajta et al. (2000) who used such dishes for the first time. The reason for a better development rate might be the microenvironment provided by small amount of medium inside the microwells, but also the macroenvironment in terms of the medium drop that connects all microwells (Vajta et al., 2000, Hoelker et al., 2010). It is very likely that the microenvironment ensured the accumulation of autocrine substances, whereby the macroenvironment provides the dilution of toxic metabolic products (Vajta et al., 2000). Also the light exposure in the camera system did not seem to have a negative effect on the development to the blastocyst stage, which is consistent with the observations in mouse embryos (Pribenszky et al., 2010a).

Compared to other authors the recovery rate of oocytes after OPU from FSH-superstimulated heifers and cows was surprisingly low (32 % vs. 46-87.7%, (Bordignon et al., 1997, van de Leemput et al., 1999, Humblot et al., 2005)). These differences in the recovery rate might be due to different superstimulation protocols but mainly caused by the performance of the OPU session. To optimize the recovery of the oocytes with expanded and sticky cumulus cells out of the follicle a flushing of the follicle like it is applied for horses would probably be useful. Furthermore, we observed that the expanded cumulus often sticks to the screen of the filter that is used for the filtration of the obtained follicle fluid. This technical problem might also result in loss of oocytes and should be optimized.

To learn more about the effect of the maturation conditions on oocyte quality and their further developmental competence, embryos after in vitro and in vivo maturation were fertilized in vitro and observed with the live observation system during a culture period of 186 hpi. At first, in vivo maturation did not seem to

have a positive effect on the embryonic development as the in vivo matured oocytes showed a significantly lower cleavage rate than oocytes after in vitro maturation (72.7% vs. 87.2%) and furthermore the blastocyst rate did not differ between both groups (43.4% vs. 44.0%). But when the blastocyst rate out of cleaved embryos was considered, the higher developmental competence of embryos from in vivo-derived oocytes became obvious. From all embryos having run through the first cleavage, the in vivo matured ones had a higher chance to reach the blastocyst stage. The higher developmental competence of embryos from in vivo-derived oocytes was especially notable in the two fold higher hatching rate compared to the in vitro matured group, although the blastocyst rate did not differ. Accordingly, the increased competence of the in vivo matured group becomes only obvious in later stages of development. The same effect could be observed regarding the kinetics: The timing of the first and the second cleavage event of embryos from in vitro or in vivo derived oocytes did not differ, but from the third cleavage on, the in vivo matured group had a significant temporal advantage.

The cleavage rate after in vivo maturation was unexpectedly low also in comparison to the results of other authors who found a cleavage rate after in vivo maturation of over 80% (Bordignon et al., 1997, van de Leemput et al., 1999, Rizos et al., 2002, Dieleman et al., 2002, Humblot et al., 2005). As the cleavage rate after in vitro maturation was in the common range (about 40%), the IVP system itself could be excluded as a reason for the low cleavage rate. More probably, the low cleavage rate could result from the low fertilization rate due to insufficient and inhomogeneous maturation of the oocytes after FSH-superstimulation and OPU. This assumption is supported by the results of Bordignon et al. (1997), who found oocytes in different stages of maturation when conducting OPU after FSH-superstimulation, namely in metaphase I and metaphase II. Furthermore, they found that an additional maturation period in vitro for 24 hours could adjust the different stages of maturation. Due to the interest for the difference in developmental competence after in vitro and in vivo maturation, additional in vitro maturation after OPU was not suitable in this experiment. To clarify the reasons for the low cleavage rate, further analysis is necessary, where the confocal microscopy of in vivo matured oocytes might be useful. Regarding the blastocyst rate, the results are in accordance to the findings of Dieleman et al. (2002), who found no difference in the blastocyst rate after in

vitro and in vivo maturation. In contrast, most of the other authors found a blastocyst rate of around 60% after in vivo maturation (Bordignon et al., 1997, Rizos et al., 2002, Humblot et al., 2005). Different results between different laboratories might also be due to differences in the superstimulation and OPU protocols, influences of the individual donors due to a low animal number, or different IVP culture systems.

A higher hatching rate after in vivo maturation is confirmed by Rizos et al. (2002). This result might be caused by faster development of the in vitro matured embryos to the blastocyst and the hatching stage, but it could also indicate that blastocysts from in vitro maturation are of lower quality and are less able to hatch than their in vivo matured counter parts. A slight developmental advance of in vivo matured oocytes regarding the blastocyst stage was also reported by Dieleman et al. (2002), as they found no difference in the total blastocyst rate after in vitro and in vivo maturation, but when they looked at the different stages of blastocysts, they observed a significantly higher percentage of expanded blastocysts after in vivo maturation. Whether the expanded blastocysts at day 8 out of in vitro maturation will still hatch or not is questionable. As the hatching of the blastocyst out of its zona is an essential requirement for the implantation into to maternal uterus, the high hatching rate after in vivo maturation might improve the pregnancy rate after transfer.

There is not much literature available about the comparison of the developmental speed of embryos from on vivo- or in vitro-derived oocytes. In my present study, the timing of the first and the second cleavage did not differ between both groups. These results are contrary to the later timing of the first cleavage after in vivo maturation compared to in vitro maturation which was observed by Rizos et al. (2002). This delayed timing of the cleavage combined with a high blastocyst rate is actually surprising and also could not be explained by the authors, since it was shown in this experiment and is also commonly accepted, that an early timing of the first cleavage is connected with a high developmental potential to the blastocyst stage. Because of the higher blastocyst rate out of the cleaved embryos and the higher hatching rate in the in vivo matured embryos, rather an earlier timing of the first and second cleavage should be expected. But it seems that the earliness of cleavage is limited. One explanation might be that there is a minimum time interval for all embryos that is required for a mitotic cleavage division, which is a summary of several cytoplasmatic and nuclear processes.

Nevertheless, there are facts suggesting a better quality of embryos after oocyte maturation in vivo: Very late cleaving embryos with nearly no chance to reach the blastocyst stage only occurred in the in vitro group, and additionally the second cleavage was less synchronous in these embryos. A higher asynchronicity of the blastomeres within one cleavage cycle after in vitro maturation might also be the reason for a delayed timing from the second cleavage on. Accordingly, the average beginning of the first and second cleavage did not differ, yet the differences in developmental speed began with an asynchronous second cleavage and were also obvious in a delay from the third cleavage on up to the expanded blastocyst stage. Until 186 hpi embryos after in vitro maturation could not compensate the delay in timing what was also visible in the significantly lower hatching rate compared to the in vivo group. A similar phenomenon was also observed in mouse embryos derived by cloning via SCNT or fertilization via ICSI (Mizutani et al., 2012). A new method of fluorescence live cell imaging allowed analyzing the reason for a delayed development of the SCNT mouse embryos compared to the ICSI mouse embryos. In accordance, the developmental delay was caused by more asynchronous blastomere cleavages within the second and the third cleavage cycle.

Another question that requires further investigation regards the contraction of the blastocoel during blastocyst stage and expanded blastocyst stage. In this experiment, the in vivo matured embryos showed this phenomenon more often. These collapses did not seem to have a negative effect on the ability to hatch or were maybe even an advantage. It is still not known, if blastocyst collapse has to be taken as a positive or negative phenomenon as there are contrary opinions. On the one hand contraction of the blastocoel was also observed in in vivo derived mouse embryos and thus thought to be physiological and necessary for the hatching process (Niimura, 2003). On the other hand less contractions were observed in human blastocysts that succeeded to hatch (Yumoto et al., 2012). In a study of Holm et al. (2002) the frequency of contractions could be influenced by the absence or addition of serum to the IVP media. As culture conditions seem to have an influence, the comparison of results might be difficult because of different IVP protocols. Whether the collapsing of the blastocoel is necessary or rather deferring for the hatching process is still not clear and need further investigations. It should be held in mind that an in vitro culture system is always

an imitation of the conditions in vivo and might therefore result in artifacts that are not comparable with the behavior of in vivo derived embryos.

2. Time lapse imaging as a tool to predict the developmental competence of in vitro produced bovine embryos

The time frames and the average times for the appearance of the cleavage events from the first cleavage until the hatching blastocyst stage were in overall consistent with the timings published by other authors (Grisart et al., 1994, Holm et al., 1998, Somfai et al., 2010, Sugimura et al., 2012). Slight differences might be caused by different culture conditions in between the laboratories. In this experiment, the timing of the first, the second and the third cleavage division was significantly later in embryos that failed to reach the blastocyst stage. This finding was absolutely in accordance with the results of other authors, who evaluated the timing of the first three cleavages by time-lapse imaging (Grisart et al., 1994, Holm et al., 1998, Somfai et al., 2010). The duration of the two-cell stage was significantly longer in embryos that arrested before the blastocyst stage. This effect was also observed in two other studies (Holm et al., 1998, Somfai et al., 2010).

In this study, the synchronicity of the second cleavage, meaning the time difference between the cleaving of first and the second blastomere, was evaluated for the first time. This required a more frequent image acquisition than in other time-lapse studies (every 5 minutes compared to 15 minutes (Somfai et al., 2010, Sugimura et al., 2012)). Accordingly, it was shown that embryos reaching the blastocyst stage had a more synchronous second cleavage division (within 36 minutes) than embryos that failed. Thus, we were able to identify and confirm a new variable for evaluating bovine embryo quality. It is recommended to use this variable to predict a successful embryo development when possible in respect to the camera system used. In addition, the optimal time interval for the first, second and third cleavage could be determined more precisely with our setup than in other studies, due to more frequently acquired images. This might be a helpful tool for the selection of embryos of good quality at the different stages.

From the 5-cell stage on until the morula stage the timing of cleavage events was not measured, since the cleavage of the individual blastomeres could not be

evaluated exactly because they were often covered by other blastomeres. This problem was also mentioned for the time-lapse imaging of human embryos (Meseguer et al., 2011). Although other authors handle this problem by estimating the blastomere cleavages by movements in the cytoplasm (Dang-Nguyen et al., 2010, Sugimura et al., 2012) this method was not used here, as it seems to be too imprecise.

When looking for variables that should prove to be useful in early embryo selection, we used logistic regression analysis. Through logistic regression analysis, variables that were highly predictive for the development to the blastocyst stage were selected. In the first model following variables were included: 1) the timing of the first cleavage (t_1), this variable was squared to consider the quadratic effect; 2) Δ_{12} meaning the time interval from the first the second cleavage; 3) Δ_{22} meaning the synchronicity of the second cleavage. Interestingly, out of several parameters that were measured, Wong et al. (2010) selected almost the same values for the prediction of the development capacity of human blastocysts as we did in our experiment. Accordingly, they also measured the time interval between the first and the second cleavage and the synchronicity of the second cleavage, but instead of the timing of the first cleavage they measured the duration of the first cytokinesis, since the time point of fertilization was unknown. In bovine embryos it is not possible to evaluate the duration of a cleavage, since due to the high lipid density in the cytoplasm, they appear so dark that the cell membranes cannot be distinguished exactly. Nevertheless, the quality of the zygotes, Wong et al. (2010) used in their experiment might be reduced by cryopreservation and as the predictive efficiency was only tested for the blastocyst rate, the results should be applied with caution for embryo selection for transfer.

In the second logistic regression model that was used for the prediction of the blastocyst stage in this experiment, the morphology of the first cleavage was included. As shown in this and other experiments, especially an unequal cleavage seems to reduce the blastocyst rate. But even if the blastocyst rate was not influenced by abnormal cleavage division in some studies (Dang-Nguyen et al., 2010, Somfai et al., 2010), these embryos should be classified as abnormal and less qualitative, since their blastocyst showed an increased percentage of abnormal chromosome number (Somfai et al., 2010, Sugimura et al., 2012), less cell numbers (Dang-Nguyen et al., 2010) and a decreased pregnancy rate (Sugimura et al., 2010, Hardarson et al., 2001) in several mammal species.

Polyspermy as a cause for abnormal cleavage was found by CLSM analyses of bovine embryos and is discussed in V.3.

Accordingly, during this doctoral thesis, a time-lapse monitoring system for bovine embryos was established for our laboratory. Based on the timing and the morphology of the first and second cleavage division, this system can be used for the selection of embryos with a high chance to reach the blastocyst stage for further experiments like the transfer on recipients or for further analyses. As a consequence of our results, we recommend using respectively adding the variables mentioned above, especially the synchronicity of the second cleavage, for a powerful time-lapse image based analysis of bovine embryos. With this tool the selection of good quality embryos for the transfer on recipients but also the selection of embryos of different quality for further analyses become possible in the future. In further analysis the cause for the delay of early cleavage divisions should be addressed.

Recent efforts were made to develop systems for automatic analysis of the time-lapse videos. There are promising results from a new software called Eeva®, that brought an improvement in the selection of embryos developing to the blastocyst stage in human embryos. Such software would be also useful and time saving for the evaluation of a high number of embryos, as it is especially necessary in basic research with animal embryos. There are no references for the application in bovine embryos, while the Eeva^{TM} software might not work appropriately, since the tracking requires high image contrast. The high cytoplasmic lipid accumulation in bovine embryos might thus hamper the correct identification of the cell membranes.

3. The meaning of polyspermy in the first mitotic cleavage division

For the evaluation of early cleavage of bovine embryos, time-lapse imaging is a useful tool, but it also has its limitations. With a light microscope the cleavage morphology can be evaluated, but the reasons for abnormal cleavage still remain unclear. For this purpose, confocal fluorescence microscopy was used to get a closer look at what happens inside the cell. As the pictures of fixated embryos are only snapshots of the mitotic cleavage process, it is necessary to collect large series of reference data in order to get an overview over normal and abnormal

cleavage. Thereby a lot of abnormal patters regarding the chromosomes and the spindle apparatus like abnormal chromosome segregation, the appearance of micronuclei, polyspermy, etc. were observed. In this work, polyspermic fertilization was used as an example to show how additional CLSM analysis can provide explanations for cleavage patterns observed during time-lapse imaging.

Polyspermy is in general a problem that is often observed during the in vitro production of mammalian embryos. To prevent polyspermy there are natural mechanisms to block the penetration of more the one sperm through the zona pellucida into the oocyte. At the current state of the scientific knowledge, the penetration of one sperm initiates the Ca^{2+} triggered plasma membrane block and the exocytosis of the cortical granules, which leads to a modification of the zona pellucida (zone hardening) called zona block. Within the last decade, the maturation of the oocyte in the oviduct was found to play an important role in a pre-fertilisation hardening of the zona pelucida (reviewed by Coy and Aviles (2010)). Under the condition of in vitro maturation the factors that are important for pre-fertilisation hardening is usually missing and thus polyspermy cannot be prevented. Accordingly, the rate of embryos fertilized with two sperms or more is higher in IVP compared to in vivo derived oocytes and ranges from 5% to 55% (Wang et al., 1997, Roh et al., 2002, Coy et al., 2005, Iwata et al., 2008, Long et al., 1993). Also in vivo derived embryos showed polyspermy although at a lower rate (18%) (Long et al., 1993). In this experiment, 26% of all embryos analyzed with the CLSM where polyspermic. The polyspermy rate can be affected by several factors including culture conditions (Iwata et al., 2008) or sperm quality of different bulls (Alomar et al., 2008).

The 3-D images of polyspermic zygotes and 2- to 4-cell stage embryos showed that an additional sperm can principally either form an additional haploid pronucleus, or the sperm head does not decondense and remains in the cytoplasm. The additional PN or sperm head can initiate a pseudocleavage, while the blastomere containing the male PN respectively the sperm head is often smaller than the other blastomere. These pseudocleavage patterns observed with the CLSM can explain abnormal cleavage morphology of the first cleavage during live observation with the camera system. Accordingly cleavage into two unequally sized blastomeres or direct cleavage into the 3- or 4-cell stage might be caused by pseudocleavage, or by the mitotic division of an additional haploid chromosome set, which results into blastomeres containing either only a sperm

head, a PN or a haploid nucleus. Pseudocleavage could also occur earlier than normal cleavage as it does not need the time span that is required for a mitotic cleavage division. This would explain why in very early cleaving embryos (before 25.6 hpi) the blastocyst rate decreased in comparison to embryos cleaving in the optimal time range. While in most studies the early timing of the first cleavage in bovine embryos is in general thought to be advantageous (Somfai et al., 2010, Sugimura et al., 2012, Grisart et al., 1994), there is also evidence in human embryos that a too early first cleavage can have a negative effect on the implantation rate (Meseguer et al., 2011).

After the first mitotic cleavage of polypermic zygotes, we found 3- and 4-cell stage embryos with diploidy or mixoploidy (both haploidy and diploidy in the same embryo). The same cleavage patterns are confirmed for porcine embryos in study of Han et al. (1999), who examined the development of porcine polyspermic zygotes with a confocal microscope. In blastocysts and fetuses derived from these zygotes, haploid, diploid, triploid and tetraploid cells were found. The origin of triploid and tetraploid cells was explained by Han et al. (1999) due to syngamy of the normal male and female pronucleus and the additional male pronucleus or pronuclei before the first mitotic cleavage. This syngamy was probably also present in our experiment as very large nuclei were observed. To prove this syngamy in bovine embryos, a differential staining of the X- and the Y-chromosome could be useful. Han et al. (1999) furthermore proved that in vitro produced porcine embryos with more than two pronuclei can result in pregnancy and the delivery of piglets. Thereby some fetuses showed haploid chromosome sets what confirms our hypothesis that the blastomere with the haploid chromosome set could arrest and the rest could progress in normal development. Nevertheless there is no definite evidence regarding the fate of polyspermic zygotes in cattle. Ulloa et al. (2008) found haploid, polyploid and mixoploid cells in bovine day-2 embryos, but mainly poly- and mixoploidy in the blastocyst stage. In consequence, they thought most of the embryos containing haploid blastomeres at day 2 to arrest before the blastocyst stage. In accordance with this Viuff et al. (2000) found di-, mixo- and polyploid cells in day 2 to day 5 embryos, but no haploid cells. Furthermore they found no polyploidy in bovine blastocysts and hypothesized that these embryos arrest before embryo-maternal transition. As a reason for polyploidy they named abnormal chromosome number in gametes, while they thought mixoploidy to be an example of polyspermy.

Although polyspermy occurs in almost all mammal animals and also in human embryos the consequence on the embryo development is not the same. Accordingly, human polyspermic embryos usually not develop to term and cause embryonic death or abortion (Coy and Aviles, 2010), while the birth of piglets after the transfer of polyspermic embryos was indeed possible (Han et al., 1999).
Until now the exact fate of polyspermic zygotes and the effect on the blastocyst rate and the blastocyst quality do not seem to be totally clear. Although the development into blastocysts and maybe even pregnancies and offspring might be possible, it is not clear how severe the effects are or if the chromosomal abnormalities can be corrected during embryo development. Since fixated embryos are only snapshots of a developmental process, the insights into the further development will be still limited. Therefore we recommend observing polyspermic but also monospermic embryos with fluorescence live imaging. As polyspermy was connected to abnormal cleavage morphology of the first cleavage in our experiment as well as in other studies (Somfai et al., 2010, Han et al., 1999), we recommend to include this into the evaluation of embryos based on time lapse imaging in future research.

VI. SUMMARY

Analysis of early bovine embryogenesis after in vitro and in vivo oocyte maturation by time-lapse imaging and 3-D confocal microscopy

In the in vitro production of embryos in humans and animals it is aimed to produce embryos of good quality in order to reach a high pregnancy rate after the transfer on a recipient. Nevertheless, data until 2007 show that in Europe the pregnancy rate after the transfer of human IVF embryos was only 33% (de Mouzon et al., 2012). Recently time-lapse imaging of early embryonic cleavage was found to be a helpful and non-invasive tool to predict the developmental capacity of embryos and select embryos of good quality (Wong et al., 2010, Sugimura et al., 2012). As the morphokinetic parameters of the first and second cleavage were the most predictive values, the fate of an embryo seems to be, at least partly, already determined very early in embryogenesis. This determination of the developmental competence might even go back further namely until oocyte maturation.

In this experiment we used bovine embryos as an animal model to study the reasons for success and failure of mitotic cleavage during early embryo development. First a live monitoring system for the observation of bovine embryos was applied in order to search for the values that are highly predictable for the developmental competence to the blastocyst stage. To consider the effect of the oocyte maturation onto the further development, we observed embryos from oocytes after in vitro (n=398) versus in vivo maturation (n=143).

In average embryos that developed to the blastocyst stage showed an earlier timing of the first, second and third cleavage than embryos that arrested ($p<0.001$). Nevertheless if the first cleavage occurred too early, the blastocyst rate also decreased. The reason for this observation is unclear and it was not described specifically for bovine embryos yet. Due to a high frequency of image acquisition, we could include the synchronicity of the second cleavage into our analyses. As already shown for human embryos we confirmed a decreasing blastocyst rate with increasing asynchronicity of the second cleavage in bovine embryos. Furthermore we observed three types of abnormal morphology during the first mitotic cleavage: 1) cleavage into two unequal sized blastomeres, 2) direct cleavage into

3- or for cells, 3) cleavage with fragments in the perivitelline space. Abnormal morphology of the first cleavage had a negative effect on the blastocyst rate as it was decreased about 25% compared to the blastocyst rate after normal first cleavage ($p<0.001$). In vitro versus in vivo maturation had no effect on the speed or morphology of the first and second cleavage. Nevertheless, from the third cleavage on until the development to the blastocyst stage we found embryos after in vivo maturation to be faster in development compared to the in vitro matured ones ($p<0.01$). Also the hatching rate was higher in blastocysts after in vivo maturation compared to in vitro maturation ($p=0.001$). Due to a logistic regression model the variables with the highest prediction rate were identified and based on two selected regression models the fate of an embryo regarding the blastocyst stage could be correctly classified in up to 76%.

To get more insights into the normal and abnormal process of the first mitotic cleavage division we fixated embryos at defined time points and analyzed them with a confocal laser scanning microscope. 3-D images of embryos showed that fertilization with more than one sperm was a main cause for abnormal cleavage. Polyspermy could result in different cleavage patterns and could partly be associated with events observed under live observation. Accordingly too early timing of the first cleavage and abnormal morphology of the first cleavage are probably mainly caused by polyspermic fertilization.

Accordingly time-lapse imaging was successfully applied to find values be able to predict the developmental competence of an embryo. In vivo maturation of oocytes had a positive effect on embryo development although this was only present from the third cleavage on. After 3-D confocal microscopic analysis, polyspermic fertilization seems to be a cause for abnormalities in timing and morphology of the first cleavage.

VII. ZUSAMMENFASSUNG

Untersuchungen zur frühen embryonalen Entwicklung des Rindes nach Eizellreifung in vitro und in vivo mittels Lebendbeobachtungen und 3-D Konfokalmikroskopie

Ein wesentliches Ziel der In-vitro-Produktion von Embryonen ist, sowohl in der Reproduktionsmedizin beim Menschen als auch in der Tierzucht, entwicklungsfähige Embryonen zu erzeugen, um eine möglichst hohe Graviditätsrate zu erhalten. Die Graviditätsrate nach Embryotransfer von humanen Embryonen nach In-vitro-Fertilisation lag in Europa bis zum Jahr 2007 bei im Durchschnitt nur 33% (de Mouzon et al., 2012). In den letzten Jahren wurde gezeigt, dass die Lebendbeobachtung von frühen Embryonalstadien eine hilfreiche und nicht invasive Methode darstellt, um die Entwicklungskapazität eines Embryos vorher zu sagen und damit entwicklungsfähige Embryonen selektieren zu können (Wong et al., 2010, Sugimura et al., 2012). Nachdem die morphokinetischen Parameter der ersten und zweiten Teilungen eine sehr hohe Aussagekraft bezüglich des Entwicklungspotentials haben, scheint es sinnvoll, auf dieser Grundlage Aussagen über das Entwicklungspotential eines Embryos zu treffen. Hierbei spielen wiederum auch verschiedene vorausgehende Faktoren wie beispielsweise die Eizellreifung, eine wichtige Rolle.

In den vorliegenden Untersuchungen wurde anhand eines Tiermodells mit Rindern nach Einflussfaktoren auf die frühe embryonale Entwicklung gesucht. Hierfür wurde zunächst ein Lebendbeobachtungssystem für Embryonen untersucht, um Parameter ausfindig zu machen, mit deren Hilfe sich das Entwicklungspotential von Embryonen bis zur Blastozyste vorhersagen lässt. Um den möglichen Einfluss der Eizellreifung auf die embryonalen Entwicklung zu bestimmen, wurden dabei sowohl Embryonen aus Eizellen nach In-vitro-Maturation (n=398) als auch nach In-vivo-Maturation (n=143) analysiert.

Die Auswertung der Zeitraffervideos zeigte, dass bei Embryonen, die sich bis zur Blastozyste weiterentwickelten, die erste, zweite und dritte Zellteilung im Durchschnitt signifikant früher stattfand als bei Embryonen, die sich nicht weiterentwickelten ($p<0.001$). Fand die erste Furchung jedoch sehr früh statt, wurde das Entwicklungspotential ebenfalls negativ beeinflusst. Die Ursachen

hierfür konnten nicht geklärt werden. Auf Grund der hohen Bildfrequenz konnte in der vorliegenden Arbeit die Synchronizität der zweiten Teilung als weiteres Qualitätsmerkmal für das Entwicklungspotential von Rinderembryonen gewertet werden. Dieser Parameter fand bis jetzt nur bei der Beurteilung von humanen Embryonen Anwendung. Es wurde nun auch für das Rind gezeigt, dass sich eine asynchrone zweite Zellteilung negativ auf die Blastozystenrate auswirkt. Die erste Zellteilung betreffend wurden drei abnormale Teilungsmuster beobachtet: 1) Teilung in zwei ungleich große Blastomere, 2) direkte Teilung in das 3- oder 4-Zellstadium und 3) Teilung mit Fragmenten im perivitellinen Spalt. Im Vergleich zu Embryonen mit einer unauffälligen ersten Zellteilung, zeigten Embryonen mit abnormalem Teilungsmustern eine signifikant geringere Blastozystenrate ($p<0,001$).

Ob die Eizellreifung in vitro oder in vivo stattfand, hatte keinerlei Effekt auf die zeitlichen und morphologischen Merkmale der ersten, zweiten und dritten Teilung. Trotzdem entwickelten sich Embryonen aus in vivo maturierten Eizellen ab der dritten Teilung deutlich schneller, was zu einer höheren Schlupfrate führte ($p=0,001$). Anhand eines logistischen Regressionsmodells wurden diejenigen Variablen bestimmt, die die höchste Vorhersagekraft besitzen, wodurch die Entwicklungskapazität eines Embryos zur Blastozyste in 76% der Fälle richtig vorhergesagt werden konnte.

Um Aussagen über den korrekten und fehlerhaften Ablauf der ersten mitotischen Teilung einer Zygote treffen zu können, wurden Embryonen zu bestimmten Zeitpunkten fixiert und mit einem konfokalen Laser-Scanning-Mikroskop analysiert. Die anschließenden, dreidimensionalen Bildanalysen zeigten, dass die Polyspermie einen wesentlichen Grund für abnormale Teilungsmuster darstellt. Diese zeigte sich in Form von unterschiedlichsten Teilungsmustern, die teilweise mit abnormalen Teilungsmustern in der Lebendbeobachtung korrelierten. Eine zu früh stattfindende erste Furchung und einige abnormale Teilungsmuster wurden vermutlich häufig durch Polyspermie verursacht.

Zusammenfassend wurden in der vorliegenden Arbeit mit Hilfe eines Lebendbeobachtungssystems erfolgreich Parameter für die Beurteilung des Entwicklungspotentials eines Embryos festgelegt. In vivo maturierte Eizellen zeigten nach Fertilisation eine bessere embryonale Entwicklung als in vitro maturierte Eizellen, was allerdings erst ab der dritten Zellteilung zu erkennen war. Durch 3-D Konfokalmikroskopie wurde gezeigt, dass die Polyspermie zu

zeitlichen und morphologischen Entwicklungsstörungen während der ersten Furchung führen kann.

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