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Morphokinetic Development Parameters of Cattle Pre-Implantation Embryos in vitro

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Abstract

Time-lapse cultivation allows the processing of the maximum amount of information on early embryo development, including such morphological disorders that are not observed in the traditional assessment of embryo cleavage at certain time intervals. The time-lapse technology was used by the authors to monitor the development of pre-implantation cattle embryos obtained from unstimulated ovaries. It was shown that the temporal and morphological characteristics of the first and three subsequent embryo divisions determine compaction probability and the embryo reaching the blastocyst stage. Embryos whose development did not fit into these time parameters did not form a blastocyst and stopped at different stages of development.

Disciplinary: Embryology

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1 Introduction

The use of high-frequency time-lapse technology for early mammalian embryo cleavage has only become possible in recent years thanks to the advent of high-tech systems built into the incubator for pre-implantation embryos' cultivation. Although time-lapse photography was first used to observe development in the 1920s [1], modern time-lapse photography systems allow obtaining high-quality images of embryo cleavage dynamics throughout the entire cultivation period without the need to move embryos from a stable incubator environment. As a result of such monitoring, it is possible to estimate the timing of the main events of early embryo development in

vitro, notice cleavage features and even predict the implantation potential of the embryo based on morphokinetic parameters [2].

The early development of human embryos has been particularly well studied, the time intervals of the first divisions have been determined, and the influence on the further development of such events as unequal cleavage, formation of cytoplasm fragments, reverse division, multi-nucleation and blastomer's division into 3 or more cells and so on has been studied. Studies on other mammals are extremely scarce. It is known that the timing of the first divisions correlates with the further embryos' development of pigs, cattle, cats, mice, and hamsters [3, 4 ,5 ,6, 7].

Observation and frame-by-frame photography of the early stages of cattle development is complicated by the high concentration of lipid granules in their oocytes and embryos, which makes it difficult to visualize pronuclei, assess fertilization, and multi-nucleation in the visible spectrum without removing the granules first [8]. The greatest difficulty is cleavage morphokinetics assessment of cattle embryos obtained from slaughter material; in this case, non-stimulated cow ovaries are used and antral follicles of different diameters from 1 to 15 mm are aspirated. The resulting oocytes are at different maturity stages and preliminary pre-incubation is required for their maturation (IVM - in vitro maturation). However, even with IVM, not all oocytes obtained from non-stimulated ovaries are potentially able to mature to MII stage, fertilize, form a zygote, and subsequently, a blastocyst. Therefore, it is interesting to evaluate the time intervals of early divisions of embryos obtained from non-stimulated cattle ovaries and to determine possible predictors of development competence to the blastocyst stage.

2 Materials and Methods

2.1 Material Sampling

After slaughter, cattle ovaries were selected and transported to the laboratory at 38.5 C in a controlled temperature environment for 4-5 hours. Immediately after receiving the ovaries, aspiration of the visualized follicles from 2 to 15 mm was performed using an 18G needle attached to a 5-10 ml syringe. Follicles' aspiration and all further work with eggs and embryos were carried out in sterile conditions of the "clean zone" in laminar cabinets with a surface heated up to 38.5°C.

2.2 Maturation and Fertilization of Oocytes, Embryos' Cultivation

Maturation of oocytes was carried out for 24 hours in a culture medium BO-IVM (IVF-Bioscience) coated with mineral oil for cell cultures (Sage) at a temperature of 38.5°C, carbon dioxide level of 6.5 vol.%, oxygen – 5.0 vol.%.

Cryopreserved bull spermatozoa frozen in 0.5 ml straws were used for in vitro fertilization. The straws were thawed in a water bath with a temperature of 37C for 30 seconds. The treatment of spermatozoa was carried out by centrifugation in a density gradient: 3 ml 80% Percoll (Irvine Scientific), for 15 minutes 400g at room temperature. After centrifugation, the sperm precipitate was washed with a buffer medium containing 3 IU of heparin for 10 minutes at 200g. After centrifugation and washing, spermatozoa were introduced into a BO-IVF (IVF-Bioscience) medium

with mature oocyte-cumulus complexes at a concentration of 1.0-2.0 x10 ^{6} motile spermatozoa in 1 ml. 18 hours after insemination, the complexes were completely purified from cumulus cells and spermatozoa and transferred to the culture medium BO-IVC (IVF-Bioscience). The embryos were cultured in vitro without medium changes for the entire development period up to the blastocyst stage.

2.3 Time-lapse Photography

The images were captured in the Primo Vision system (Vitrolife, Sweden) for 8 days (192h) after the fertilization of mature cattle oocytes. The Primo Vision device is a compact digital inverted microscope with integrated optics, Hoffman contrast and green LED illumination (550 nm), which is placed in a conventional laboratory incubator and communicates with a control unit located outside the incubator. Special cups designed for this system were used for cultivation, which can accommodate up to 16 embryos placed in 4 rows of 4 drops. The system was configured to receive 1 photo every 10 minutes. All received images were saved for further analysis.

3 Results

A total of 5 series of time-lapse photography were recorded, 50 dividing embryos obtained during the maturation and fertilization of non-stimulated cattle oocytes were filmed. The level of blastocyst formation amounted to 22%, the remaining embryos stopped developing at different stages.

The following time parameters were evaluated:

- the duration of the first division from the moment of the cleavage furrow appearance (Figure 1A) to the end of cytokinesis and the formation of a 2-cell embryo (Figure 1B);
- the time of the first division from the moment of fertilization to the formation of a 2-cell embryo;
- the time of the second embryo division from the moment of fertilization to the formation of a 3-cell embryo (Figure 1C);
- the time of the third embryo division from the moment of fertilization to the formation of a 4-cell embryo (Figure 1D);
- the time of the fourth division from the moment of fertilization to the formation of a 5-cell embryo (Figure 1E);
- time of blastocyst formation (blastulation) (Figure 1G).

A B C

(Figure 1 continues on the next page).

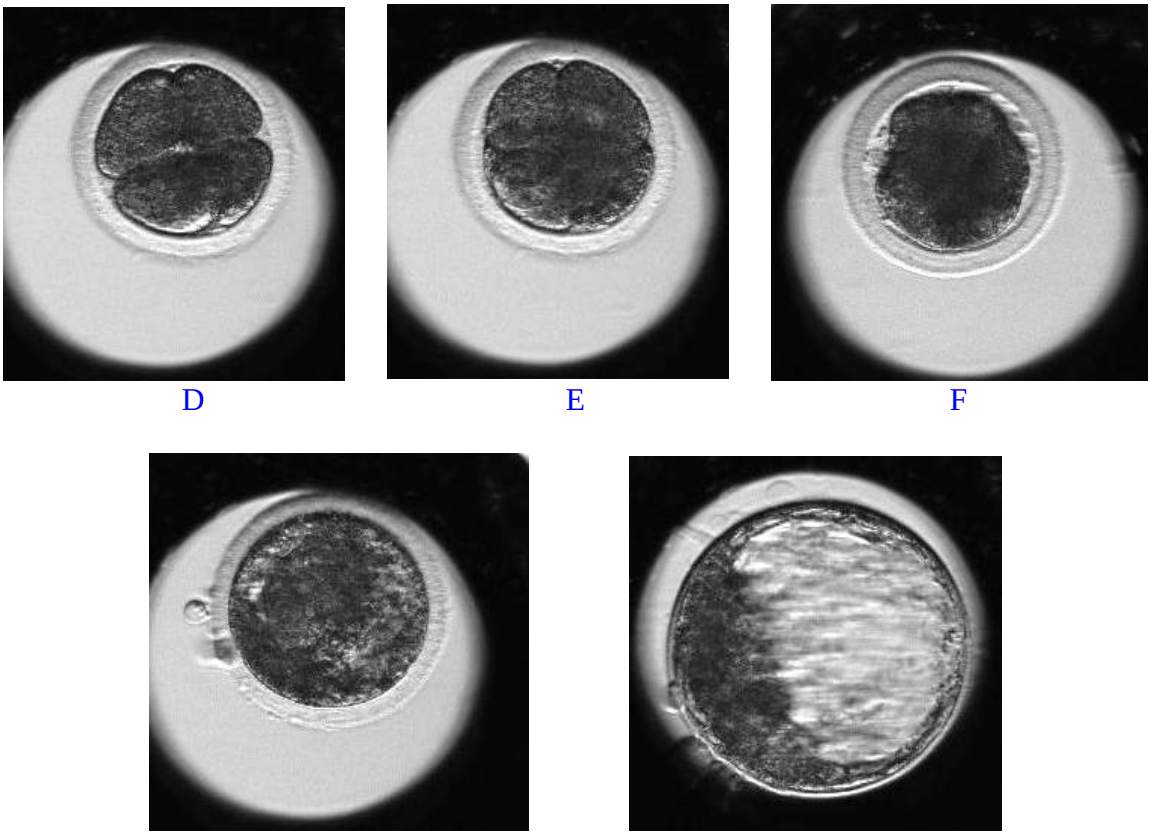


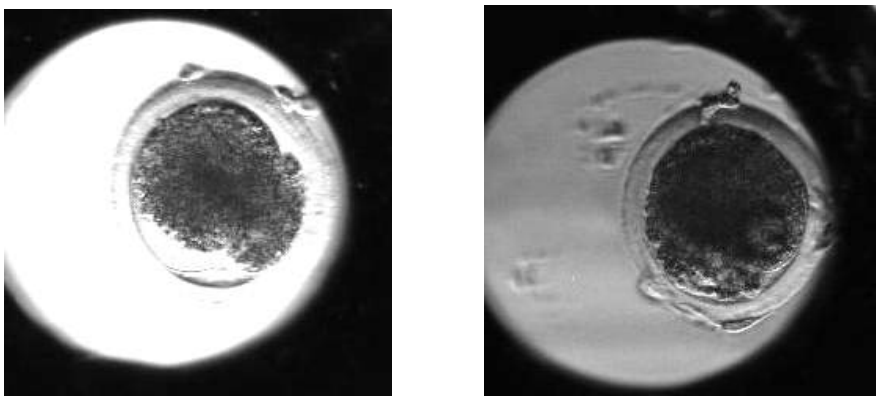
Figure 1: Normal development of the pre-implantation cattle embryo. A-the appearance of the first mitotic cleavage furrow; B-the end of the first mitotic division, the formation of a 2-cell embryo; C-3-cell embryo; D-4-cell embryo; E- 5-cell embryo; F-blastomeres compactification, morula formation; G-the beginning of blastulation; H-expanded blastocyst.

Table 1: Morphokinetic indicators of cattle embryos' development obtained from non-stimulated cattle follicles.

indicator	Embryos developed to blastocysts	Embryos arrested in development	Mann-Whitney Test	t-test
Total q-ty	11	39		
First division, h	27,08 ± 0,81	32,81 ± 6,71	0,0007	0,007
Duration of the first division, min	13,41 ± 3,56	16,41 ± 2,46		
second division, h	35,87 ± 1,16	43,27 ± 6,40	0,0004	0,005
Interval from 2-cell to 3-cell embryo, h	8,37 ± 1,25	11,60 ± 4,20	0,005	0,017
Third division, h	38,33± 2,83	44,93 ± 8,47	0,005	0,017
Interval from 3-cell to 4-cell embryo, h	2,40 ± 0,90	2,99 ± 1,01		
Fourth division, h	44,81 ± 1,35	53,37 ± 3,63	0,001	
Interval from 4-cell to 5-cell embryo, h	5,65 ± 2,07	11,67± 3,28	0,004	
Interval from 5-cell to blastulation, h	122,07 ± 10,82			
Interval from fertilization to blastulation, h	173,0 ± 10,9			
Oocyte size, microns	151 ± 0,8	151 ± 0,8		

The blastomer's division into two equal parts without fragmentation signs was assessed as normal (Figure 1). If two blastomeres of unequal size were formed during division (Figure 2A), or nuclear-free fragments of cytoplasm were formed in the perivitelline space (Figure 2B), or the

blastomer was split into 3 or more parts (Figure 3A-B), then the division was considered abnormal. Unequal mitotic division was found in 12% of embryos, blastomer division into more than two parts - in 6% of embryos, and fragmentation - in 4% of embryos.



A B

Figure 2: A- unequal first division with the formation of blastomeres of different sizes. B-formation of cytoplasmic fragments.

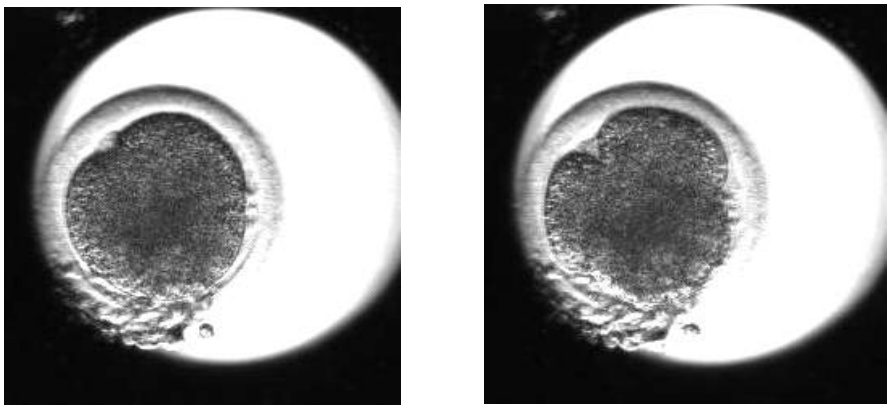


Figure 3: Division of the embryo into three blastomeres: A-the formation of the division furrow, B-the end of cytokinesis

Abnormal development features included recursive division-reabsorption cases of blastomeres or large cytoplasm fragments (Figure 4) (8% of embryos), as well as cases of blastomeres' exclusion from compaction and blastocyst formation (Figure 5) (6%).

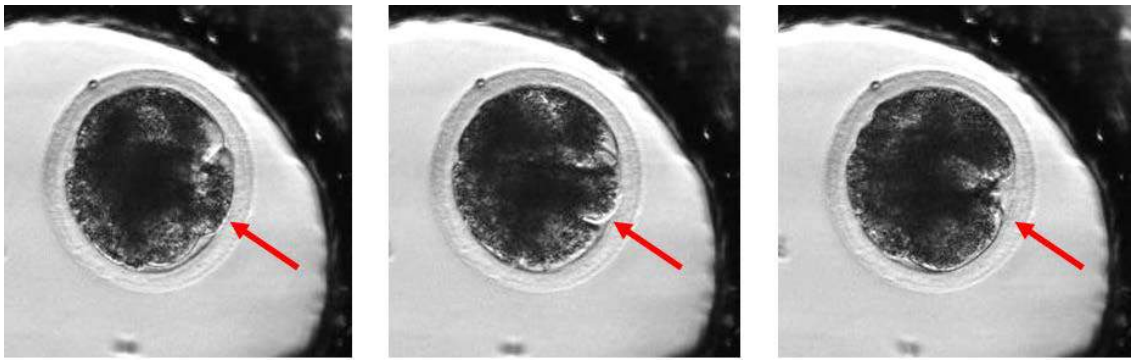


Figure 4: Reversive division.

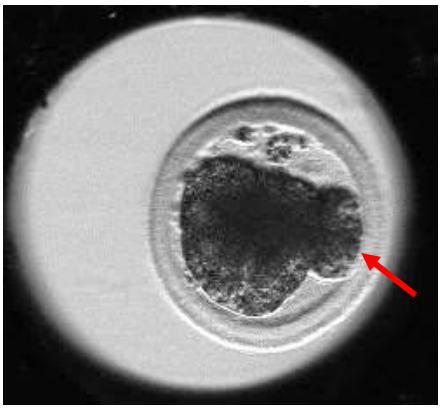


Figure 5: In the morula stage, one of the blastomeres is excluded from compaction.

4 Discussion

Time-lapse cultivation allows processing the maximum amount of information on the development of pre-implantation embryos, including morphological disorders that are not observed in the traditional assessment of embryo cleavage. Figure 1 shows the stages of the first cattle embryos' mitoses, while all divisions occur without the occurrence of cytoplasm fragmentation and the embryo forms an expanded blastocyst on the 8th day of development. The duration of the first cell cycles (time intervals of the first and three subsequent divisions of the embryo) determines the compaction probability and the embryo reaching the blastocyst stage (Table 1). Embryos which development did not fit into these parameters did not form a blastocyst and stopped at different stages of development. One of the reasons for such delayed cleavage may be multiple chromosomal pathologies; so, in human embryos and primates, it has been shown that the frequency of chromosomal abnormalities is significantly higher in slowly dividing embryos [9, 10].

According to the results, in cases where the embryo forms more than one cleavage furrow and splits into three or more blastomeres, the probability of blastocyst formation is very low, which is consistent with the data of other authors obtained on pre-implantation human embryos. Such cytokinesis anomalies are caused by disorders of the spindle and incorrect chromosome divergence [11]. If such mitotic disorders occur at the very first mitotic division, the embryo carries numerous chromosomal abnormalities that lead to the arrest of its development [12].

The most common anomaly observed in human IVF embryos is cytoplasmic fragmentation. This indicator is considered in all classification systems and is a key parameter determining the quality of embryos since it is known that the presence of fragmentation reduces the viability of embryos and the likelihood of their implantation into the uterus [2]. However, according to the authors' data, fragmentation was extremely rare in cattle embryos - only in 4% of cases. More often, fragmentation occurred repeatedly in the same embryo; but the greatest impact on development was the fragmentation that occurred during the very first embryo's mitosis. During cytoplasm fragments' formation at later stages of development (6-8 cell embryo), their presence did not

prevent the further development of the embryo to the blastocyst stage. Moreover, during the formation of the morula, parts of the cytoplasm fragments did not participate in compactification and were excluded from further embryo development. Sugimura et al. obtained cattle embryos after hormonal stimulation of the ovaries and observed a higher potential for implantation into the uterus in embryos with correct mitotic division (into two blastomeres of equal size without fragmentation) (66.7%) than in embryos with abnormal division (33.3%) [13]. Similar data were obtained on human embryos and primates [14,15].

Two other important parameters are cytoplasmic vacuoles and multi-nucleation of blastomeres. Multi-nucleation – the presence of more than one nucleus in the blastomere - is one of the signs of chromosomal abnormalities in the embryo. However, this indicator cannot be tracked in cattle embryos either by conventional microscopy or by time-lapse due to the abundance of lipid granules in the cytoplasm.

Vacuoles in the cytoplasm appear either as a result of vesicles' fusion from the Golgi apparatus, or smooth endoplasmic reticulum, or are formed spontaneously [16]. The presence of vacuoles in the cytoplasm is an unfavorable prognostic trait since it can lead to the displacement of the meiotic spindle and disruption of the cytoskeleton; it also indicates the processes of apoptosis in the embryo [17]. In this study, there were no cattle embryos with vacuolization at early cleavage stages.

Thus, slowly dividing embryos had low viability and low potential for blastocyst formation. Conversely, viable embryos had shorter cell cycles. The authors' data are consistent with Sugimura data obtained on cattle embryos after hormonal ovarian stimulation [18].

5 Conclusion

Time-lapse cultivation allows the processing of the maximum amount of information on early embryo development, including such morphological disorders that are not observed in the traditional assessment of embryo cleavage at certain time intervals. This study used the time-lapse technology to monitor the development of pre-implantation cattle embryos obtained from unstimulated ovaries. It was shown that the temporal and morphological characteristics of the first and three subsequent embryo divisions determine compaction probability and the embryo reaching the blastocyst stage. Embryos whose development did not fit into these time parameters did not form a blastocyst and stopped at different stages of development.

6 Availability of Data and Material

Data can be made available by contacting the corresponding author.

7 Acknowledgments

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