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Time of early cleavage affects the developmental potential of feline preimplantation embryos *in vitro*

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ABSTRACT

The study compared developmental competence of embryos, based on the timing of the first cleavage and morula formation, with their subsequent ability to reach the blastocyst stage and the resulting blastocyst morphology and quality. Cleaved embryos were separated at 18 hours post insemination (hpi), 24 hpi, and 30 hpi and then cultured in droplets to follow the individual embryo development. The significantly higher percentage of the blastocyst formation was noted in the group I of embryos cleaving within less than 18 hpi (12.7%) compared with the group II cleaving within 18 to 24 hpi (10.7%), None of the late-cleaving embryos (group III >24–30 hpi) reached blastocyst stage in our experiment. Interestingly, the hatching ability was similar regardless the time of the first cleavage (I: <18 hpi; 6.4% and II: 18–24 hpi; 5.4%). The ability to hatch was correlated with the time of morula formation; only embryos that reached morula on Day 4 or Day 5 were able to develop into hatching blastocyst (8.4% and 3.3%, respectively). The differential cell staining revealed significantly more blastomeres in blastocysts obtained from embryos cleaving within 18 to 24 hpi than the blastocysts obtained from embryos cleaving less than 18 hpi (188.6 ± 21.9 vs. 129.3 ± 16). Embryos cleaving within 18 to 24 hpi also demonstrated the higher number and percentage of embryoblast cells (97.2 ± 12.6, 51.6 ± 2.9 vs. 46.6 ± 9, 36.3 ± 6.6). The presented results confirmed the association among the onset of the first cleavage, time reaching morula, and subsequent blastocyst formation and quality.

1. Introduction

The identification of an embryo with the highest developmental potential is of major importance during *in vitro*-assisted reproduction techniques. Many methods have been proposed to evaluate embryo quality during *in vitro* culture. Among the noninvasive methods, the most common used is the standard morphologic evaluation. However, the outer appearance might not always reflect the embryo's developmental potential [1–3]. The influence of the time of the first cleavage on the pregnancy rate has been reported in mice [4], humans [5,6], bovine [7], and ovine [8]. In humans, a distinct association has been found between the time of the completion of the first cell cycle and the blastocyst development. Hence, in human preimplantation embryos, it was recommended to use the timing of the first cleavage as an additional marker of their viability and a strong prognostic factor for further transfer outcomes [9,10].

Because, most of the wild felid species are classified as vulnerable or endangered, the domestic cat can be used as a research model for reproductive studies in Felids. The assisted reproductive techniques developed for domestic cats have successfully been applied in leopard cat, Indian desert cat, tigers, or cheetah [11–14]. Nevertheless, the *in vitro* development to the blastocyst stage is still reduced and delayed relative to *in vivo* cat embryos, and the pregnancy rates after embryo transfer are reported to be lower

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compared with natural mating. Similarly, embryo transfer success is considerably lower in cats than in other species [15–17]. Although, the chronological events of cat embryo development are known, the data regarding feline embryo physiology, viability, and developmental potential is still limited. During *in vitro* culture, the most commonly applied embryo selection is the morphologic assessment. Recent studies suggested it might not be sufficient to accurately predict further performance of embryos, and indicated that the timing of the first cleavage might be related to the subsequent developmental potential [3]. Klincumhom [18] recommended the first cleavage time as a reliable, simple, and noninvasive prognostic tool for the selection of embryos with the highest capability of implantation. Moreover, pronuclear stage kinetics and morphology would be useful in transfer protocols of cleavage state embryos [19].

Since the blastocyst is the last embryo developmental stage taking place within the zona pellucida, it is also the final point for an *in vitro* culture. Its viability and potential depend on the intrinsic embryo quality and culture system used [15]. One of the most common methods to evaluate blastocysts is differential cell staining. This method allows for differentiation between inner cell mass (embryoblast, ICM) and trophoblast cells (TE). Allocation of the sufficient number of cells to both the lineages is imperative for normal preimplantation development [7]. To differentiate between these two cell lineages, bichromatic fluorochrome staining and direct cell visualization are usually applied [20,21].

The overall goal of this study was to evaluate the possible association among the time of first cleavage, feline embryo developmental kinetics and its subsequent ability to reach the blastocyst stage, and the resulting blastocyst morphology and quality.

2. Materials and methods

Unless otherwise stated, all chemicals and reagents used in this study were purchased form Sigma-Aldrich, Poland.

2.1. *Oocytes collection and* in vitro *maturation*

Ovaries were collected from sexually matured queens (n = 116) during routine ovariectomy or ovariohysterectomy and immediately stored in a transport medium (PBS supplemented with 1% of antibiotic antimycotic solution) at 4 °C. They were kept no longer than 24 hours until oocytes recovery. Cumulus oocyte complexes were released by slicing ovaries with a scalpel blade in washing medium (WM) containing medium 199 with Earle's salts, supplemented with 3 mg/mL BSA, 0.1 mg/mL cysteine, 1.4 mg/mL HEPES, 0.25 mg/mL sodium pyruvate, 0.6 mg/mL sodium lactate, 0.15 mg/mL L-glutamine, and 0.055 mg/mL gentamicin [22]. For *in vitro* maturation, only oocytes with regular dark ooplasm pigmentation and several layers of cumulus cells were selected and were placed in 400 μL of maturation medium (WM with addition of 0.025 IU/mL LH and 0.02 UI/mL FSH) under mineral oil and matured for 24 hours at 38.5 °C in 5% CO_2 in air with maximum humidity.

2.2. In vitro *fertilization and* in vitro *culture*

For *in vitro* fertilization, thawed spermatozoa isolated from cauda epididmis and frozen according to the procedure described by Niżański [23] were used. After 24 hours maturation, cumulus oocyte complexes were washed twice in WM and coincubated for 18 hours with 1×10^6 motile spermatozoa/mL at 38.5 °C in 5% CO_2 in air with maximum humidity, in Tyrode's salt solution supplemented with 6 mg/mL BSA, 1.2 mg/mL HEPES, 1.1 mg/mL sodium lactate, 0.15 mg/mL L-glutamine, and 0.1 mg/mL sodium pyruvate. Embryos were cultured up to 9 days in individual droplets of 20 μL of pre-equilibrated culture medium under mineral oil (MEM Eagle supplemented with 0.055 mg/mL gentamicin, 0.3 mg/mL sodium lactate, 0.15 mg/mL sodium pyruvate, 0.5 μL/mL MEM Non-Essential amino acid solution 100×, 0.04 mg/mL BSA for first 48 hours of culture, and after with 10 mg/mL fetal bovine serum for the rest of the culture) [24]. Embryo development was assessed based on the morphologic characteristics. Blastocyst had the blastocoele cavity at least one third of the embryo, whereas hatching ones had obvious TE cells emerging through zona pellucida.

2.3. *Differential cell staining and counting of the nuclei*

Expanded blastocysts were stained to asses total cell number (TCN), TE cells and ICM using protocol described by Thouas [20] with slight modifications [25]. Briefly, zona-intact blastocyst were incubated for 15 seconds in PBS containing 0.5% Triton-X and 100 μg/mL propidium iodide in 38 °C to stain TE cells. Blastocysts were then immediately transferred into 100% ethanol with 15 μg/mL Hoechst 33342 and incubated for at least 40 minutes in 4 °C to stain ICM. Cells were mounted onto a glass microscope slide with a drop of glycerol, flattened with a coverslip, and evaluated using epifluorescence inverted microscope (Olympus IX 73) with UV excitation of 345 and 535 nm resulting in bisbenzimide-stained nuclei fluorescing blue (ICM), whereas propidium iodide-stained cells fluorescing red (TE). Normal nuclei displayed distinct nuclear outline and even shape and color. Photographs were taken and the number of TE and ICM were counted using Olympus cellSens Dimension software. Number of blastomeres and their allocation were estimated based on the assumption that they are equivalent to the number of nuclei counted. The ICM ratio was counted as the proportion of the total number of blastomeres.

2.4. *Experimental design*

The principle of these investigations was to determine the time of first cleavage and the morula formation in relation to the further ability of the embryo to reach blastocyst and its morphology. For each replicate of the study, 20 ± 5 oocytes were matured *in vitro* (40 replicates) and then fertilized. Embryo development was assessed based on morphologic features. In the second part of the experiment, differential cell staining was performed for expanded blastocysts (n = 20) divided into three groups, based on the time of the first cleavage (group I: <18 hours post