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CAT PRESUMPTIVE ZYGOTES ASSESSMENT IN RELATION TO THEIR DEVELOPMENT*

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Abstract

The evaluation of oocytes and zygotes, based on their size, shape and morphology, is a valuable tool for predicting subsequent embryo development. While this assessment is non-invasive and made possible with time-lapse monitoring systems, not all the assessment criteria used for zygotes with pale cytoplasm can be used for domestic cat zygotes, which have dark cytoplasm. In this study, feline presumptive zygotes were evaluated for shape, size, and morphology. Measurements were also made of the diameter of the entire zygote, its cytoplasm, and the zona pellucida. Differences in the dataset were assessed using the generalized linear model (GLM) procedure. While there was no relationship between a combination of the tested parameters with the potential for cleavage, blastocyst development, and hatching, the parameters of the shape and size of the entire oocyte, and of the zona pellucida, were related to the development potential. The results presented in this study indicate that the assessment procedure for human zygotes has to be adjusted to be used in the cat model, however the relationship between measurements of the diameter of presumptive feline zygotes and the thickness of zona pellucida with their developmental potential deserves further investigation to optimize assessment of cat presumptive zygotes.

Key words: domestic cat, presumptive zygotes, zygote morphology, embryo development

The effectiveness of assisted reproductive techniques used in felids is particularly low and requires continuous improvement (Kochan et al., 2021 a). Prediction of embryo development is extremely important in the context of determining their developmental and implantation competencies. One of the first evaluation elements is oocyte and/or zygote assessment which is an important element in predicting their subsequent development and implantation potential. This assessment consists of determining the oocyte size and shape, the quality of the cytoplasm both in the oocyte and the subsequent zygote, as well as extracytoplasmic features such as the thickness and adhesion of the zona pellucida or the perivitelline space (Esfandiari et al., 2005; Ebner et al., 2008; Ozturk, 2020; Bassil et al., 2021). In addition, the number, size and shape of pronuclei are assessed in human, mouse, and bovine zygotes (Krukowska and Tarkowski, 2005; Araki et al., 2018; Suzuki et al., 2021).

Abnormal human oocyte size is correlated with abnormal oocyte ploidy. For example, oocytes that are too large (known as giant oocytes) are considered tetraploid, resulting from abnormal cell division or the fusion of two oogonia (Machtinger et al., 2011). Although variations in oocyte shape are not related to fertilization competence or embryo quality, embryos formed from such oocytes show delayed development (Ebner et al., 2008).

Another element in oocyte evaluation is the cytoplasm, which should be characterized by a uniform color, good turgor and the absence of cytoplasmic vacuoles or granularity. The presence of these deviations might interfere with the cell division process, resulting in a reduced number of blastocysts and lower developmental or implantation potential (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011).

Variations in the zona pellucida of human embryos are related to disorders in the secretion and modeling of the glycoprotein matrix which are closely related to the possibility of sperm penetration (Töpfer-Petersen et al., 2000; Wassarman, 2008; Accogli et al., 2014).

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In addition to the parameters assessed for oocytes, zygote assessment also includes evaluation of the pronuclei. Abnormalities in the number and morphology of these structures are closely correlated with embryo cell division arrest and chromosomal abnormalities (Coskun et al., 2003; Brooks et al., 2022; Hatano et al., 2022). Detailed observation and analysis of each of these morphological elements in oocytes and zygotes is possible due to the use of time-lapse systems for continuous observation of embryo development. These systems allow the continuous and uninterrupted observation of cell division, and the analysis of morphokinetic parameters (Kochan et al., 2022; Kij-Mitka et al., 2022). The use of time-lapse systems for the analysis of animal embryos characterized by light cytoplasm does not differ from human clinical embryology (Mateusen et al., 2005; Pribenszky et al., 2010; Kirkegaard et al., 2012).

However, despite the variety of possibilities offered by such systems, in the case of feline oocytes and zygotes, which are characterized by a dark cytoplasm, these possibilities are limited. In this situation, the non-invasive assessment of oocytes and zygotes focuses on the size and shape of the oocyte, the normality of the cytoplasm and the zona pellucida. Due to the impossibility of observing pronuclei in zygotes with a dark cytoplasm, they are called presumptive zygotes (PZs), with no certainty about the oocyte fertilization status.

This study was designed to investigate the relationship of three parameters from PZ assessment (i.e. shape and size, cytoplasmic morphology, zona pellucida morphology) with their competence for subsequent embryo development (i.e. cleavage, blastocyst formation and hatching) in the domestic cat.

Material and methods

Experiments were conducted according to the guidelines approved by the Local Ethics Committees for Animal Experimentation in Wrocław (106/2014 II Local Ethics Committee in Wrocław).

Collection of cumulus-oocyte complexes

The research material consisted of ovaries obtained from 50 adult female cats aged approximately 1–2 years, isolated after routine ovariohysterectomy. The ovaries were transported to the laboratory in PBS up to 24 h after castration. Cumulus-oocyte complexes were obtained from the ovaries after scarification of the ovarian cortex in TCM 199 medium with the addition of 10% bovine serum, according to the procedure previously described by Kij et al. (2020).

Oocyte *in vitro* maturation (IVM)

Oocytes characterized by a dark cytoplasm were transferred to a 4-well dish filled with 400 µl BO-IVM (IVF-Bioscience, Great Britain) and incubated for 24 h at 38°C under a humidified atmosphere of 5% CO_2. Cumulus–oocyte complexes after IVM were partially denuded for 2 min in TCM 199 + 10% FBS with hyaluronidase (80 UI/ml) using a glass capillary. Oocytes surrounded by corona radiata cells were transferred to a 4-well dish filled with 400 µl BO-IVC medium (IVF-Bioscience, Great Britain).

In vitro fertilization (IVF)

Metaphase II oocytes with visible first polar body were inseminated with 5×10^6 capacitated spermatozoa/ml, using sperm from the Wrocław University of Environmental and Life Sciences, Department of Reproduction and Clinic of Farm Animals sperm bank, prepared according to methodology described by Kij-Mitka et al. (2022). Oocytes were incubated with sperm for 16 h at 38°C in 5% CO_2 atmosphere.

In vitro culture of embryos in the time lapse system

After 16 h incubation, 16 PZs were washed in BO-IVC medium (IVF-Bioscience, Great Britain) to remove excess spermatozoa and randomly transferred in a Primo-Vision® culture dish (Vitrolife, Sweden) filled by BO-IVC medium (IVF-Bioscience, Great Britain). Culture dish was prepared in advance according to the manufacturer's instructions. The development of the embryos was photographed at 10-minute intervals for about 8 days. In order to ensure appropriate conditions for the development of embryos, the medium was partially replaced every other day (Kij-Mitka et al., 2022).

Morphology analysis of PZs

Images of PZs taken after 19 h post fertilization were evaluated for the following morphological parameters:

– size of PZ (AD1 – average diameter) (Figure 1B), cytoplasm (AD2) (Figure 1C) and zona pellucida (AD3) (Figure 1 D),
– shape of PZ (M1 – morphology) (Figure 2),
– morphology of cytoplasm (M2) (Figure 3),
– morphology of zona pellucida (M3) (Figure 4).

In order to measure the diameter of the entire PZ, cytoplasm and zona pellucida, two measurements were made using ImageJ software (1.8.0) (Figure 1 B–D). The scale used for defining the measurements was the diameter of the well (240 µm) (Figure 1 A). PZs with a total diameter, cytoplasm diameter, and zona pellucida diameter that were in the range of 143.96–178.79 µm, 93.17–118.35 µm and 21.16–31.75 µm, respectively, were considered to be normal within this parameter. The ranges were determined by calculating the mean in the study population ± standard deviation. In addition, deviations from the correct shape (i.e., other than round) were assessed (Figure 2 A).

PZs with a uniform color, good turgor and lack of cytoplasmic vacuoles or granularity were defined as exhibiting normal cytoplasmic morphology. Deviations in the cytoplasm morphology are shown in Figure 3. In the case of the zona pellucida, shape, size and homogeneity were

evaluated. Examples of disorders within zona pellucida are presented in Figure 4. Assessment and measurement of each PZ were performed by two independent investigators. Normal PZs were considered to be those with a size, shape, and morphology that met the established standards.

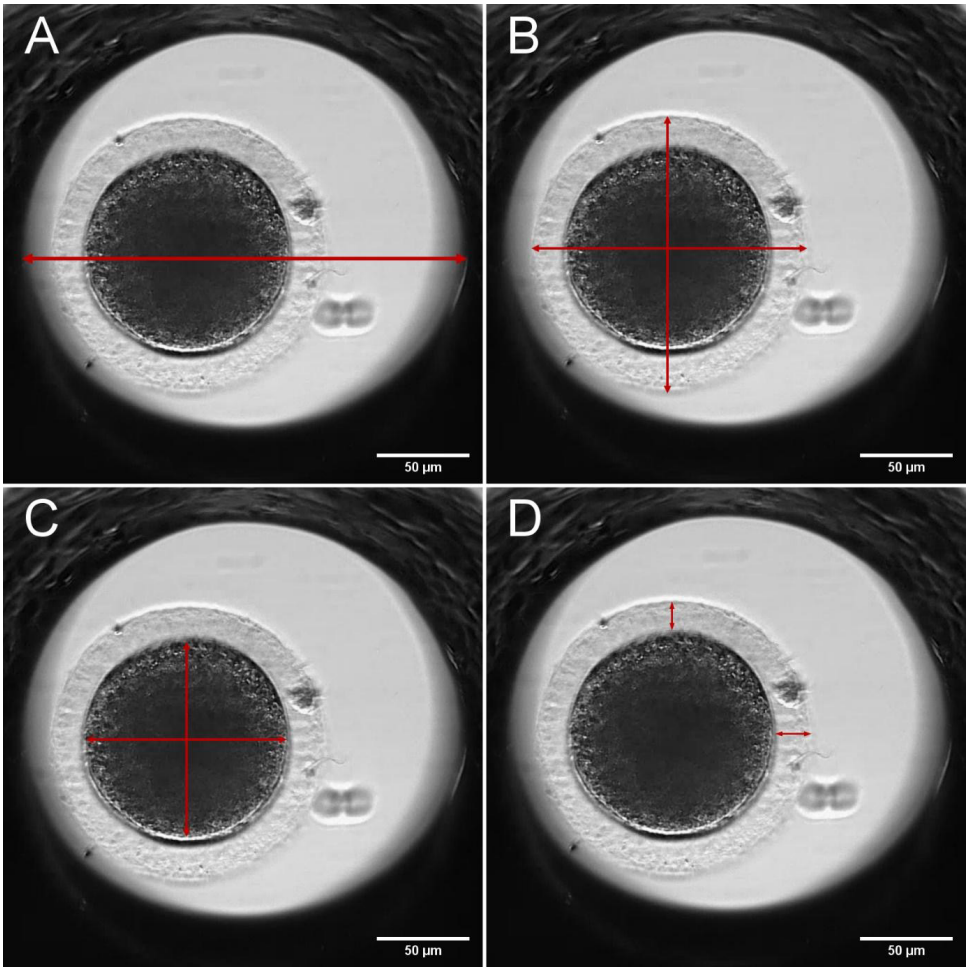


Figure 1. Measurement of the diameter of the bottom of the well (A), whole domestic cat presumptive zygote (B), ooplasm (C) and zona pellucida (D)

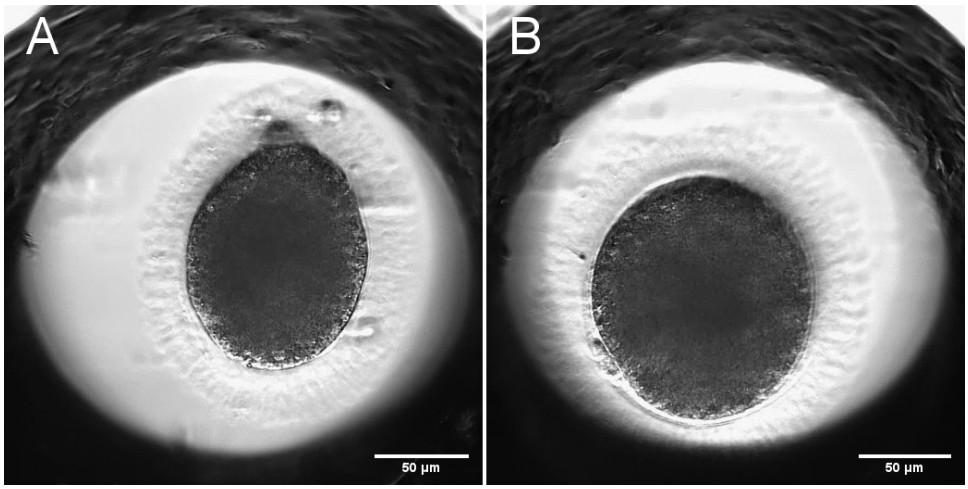


Figure 2. Examples of abnormalities in size and shape of domestic cat presumptive zygotes: oval shape (A), giant oocyte (B)

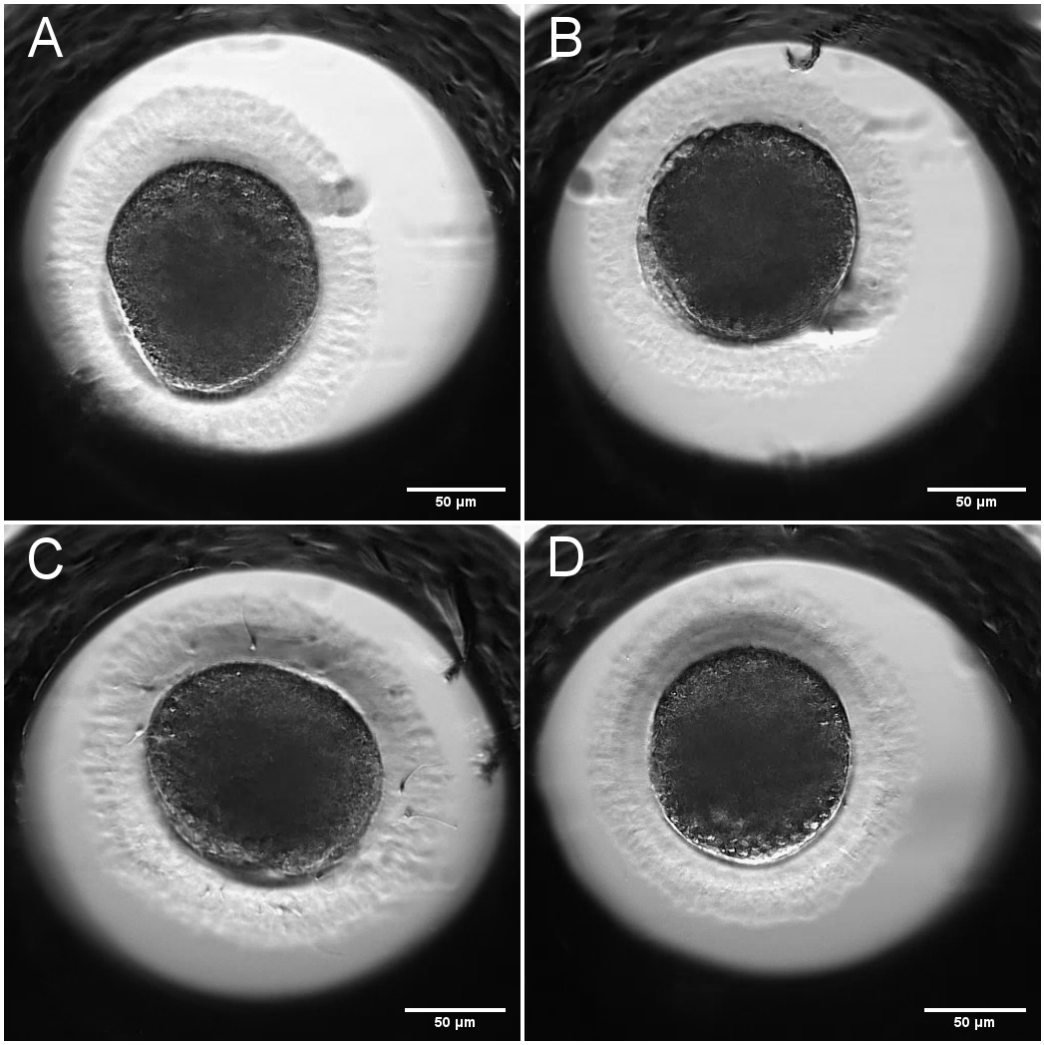


Figure 3. Examples of cytoplasmic abnormalities of domestic cat presumptive zygotes: irregular shape (A), partial fragmentation (B), low turgor (C), vacuoles (D)

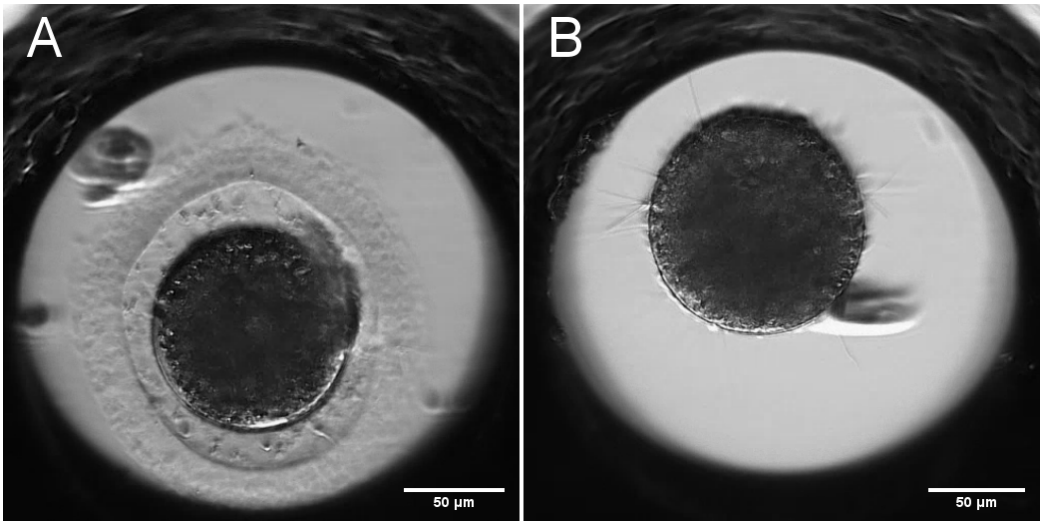


Figure 4. Examples of zona pellucida abnormalities of domestic cat presumptive zygotes: non-adherent (A), lack of zona pellucida (B)

Development potential assessment

The factors included in the assessment of the developmental potential of the presumptive zygotes were the occurrence of the first embryonic cleavage, blastocyst development (defined as the presence of a blastocoel), and the ability of the blastocyst to hatch.

Statistical analysis

All of the statistical analyses were performed using R software (version 4.2.2) supported by the Agricolae package. The generalized linear model (GLM) procedure was used to assess the differences in the dataset ($P<0.05$). Duncan test was used for mean comparisons. The number of embryos, blastocysts and hatching blastocysts was compared using Student's t-test.

Results

Of the 336 PZs obtained in 21 replicates, 287 were analyzed, while 49 (15%) PZs were excluded from the analysis due to inadequate image quality or evidence of early cleavage. The mean diameter of the presumptive zygotes was 161.37±17.42 µm. The average diameters of the cytoplasm and zona pellucida were 105.76±12.59 µm and 26.45±5.29 µm, respectively. Of the 287 PZs, 67 (23.34%) showed cleavage, 27 (9.41%) reached the blastocyst stage, and 16 (5.57%) started hatching. The number of embryos, blastocysts and hatching blastocysts relative to the diameters of the entire presumptive zygote, cytoplasm, and zona pellucida are presented in Table 1. Additionally, Table 2 presents results of developmental competence of presumptive zygotes with regular and irregular shape. The number of embryos developed from regular and irregular shape PZ differed for $P = 0.000319$. Additionally, the number of blastocysts and hatching blastocysts in compared group did not differ ($P = 0.050948$ vs. $P = 0.5$).

There was no statistically significant relationship between AD1, AD2, AD3, M1, M2, and M3, combined, and the potential for the presumptive zygotes to cleave, reach blastocyst stage and/or hatch. However, when assessing the association of each individual parameter with the potential of a presumptive zygote to cleave, reach the blastocyst stage, and hatch, there were statistically significant differences for AD1 ($P=0.007$) and for AD3 ($P=0.039$). There was also a statistically significant relationship between AD3 and the potential to hatch ($P=0.019$).

Table 1. Number of embryos, blastocysts and hatching cat blastocysts relative to the diameters of the entire presumptive zygote, cytoplasm, and zona pellucida. The measurements were performed on images of presumptive zygotes taken after 19 h post fertilization. The scale used for defining the measurements was the diameter (Ø) of the well (240 µm). Cat presumptive zygotes with a total diameter, cytoplasm diameter, and zona pellucida diameter that were in the range of 143.96–178.79 µm, 93.17–118.35 µm and 21.16–31.75 µm, respectively, were normal within this parameter. The ranges were determined by calculating the mean (Ø) in the study population ± standard deviation (SD)

	Whole presumptive zygote average diameter N (%)			Cytoplasm average diameter N (%)			Zona pellucida average diameter N (%)		
	Ø < 143.95 µm	Ø = 161.37 ± 17.42 µm	Ø > 196.21 µm	Ø < 93.2 µm	Ø = 105.76 ± 12.59	Ø > 118.35 µm	Ø < 21.16 µm	Ø = 26.45 ± 5.29 µm	Ø > 31.74 µm
Embryos N=67	5 (7)	59 (88)	3 (5)	2 (3)	63 (94)	2 (3)	10 (15)	53 (79)	4 (6)
Blastocyst N=27	3 (1)	24 (99)	0 (0)	1 (4)	26 (96)	0 (0)	6 (22)	19 (71)	2 (7)
Hatching blastocyst N=16	3 (19)	13 (81)	0 (0)	0 (0)	16 (100)	0 (0)	6 (37)	10 (63)	0 (0)

Table 2. Number of embryos, blastocysts and hatching cat blastocysts relative to the shape of the entire presumptive zygote. The assessment was performed on images of presumptive zygotes taken after 19 h post fertilization. All presumptive zygotes with shape other than round were assessed as irregular

	Regular shape of presumptive zygote N (%)	Irregular shape of presumptive zygote N (%)
Embryos N=67	51 (76) a	16 (24) b
Blastocyst N=27	18 (67) a	9 (33) a
Hatching blastocyst N=16	8 (50) a	8 (50) a

Values with different letters (a, b) differ between the groups ($P<0.05$).

Discussion

The domestic cat (*Felis catus*) is used as a model animal for endangered wild felids. Due to the extremely valuable gametes from cats of the *Felidae* family, experimental research in the field of embryology and assisted reproduction is conducted on material from domestic cats (Kij et al., 2019). Most commonly only 50% of oocytes will reach the metaphase II stage *in vitro*, of which 50% will exhibit cleavage (Freistedt et al., 2001 a). Moreover, only 20% of embryos reach the blastocyst stage (Freistedt et al., 2001 a; Gómez et al., 2003; Thongkittidilok et al., 2015). One of the first evaluation elements is oocyte and/or zygote assessment, which focuses on the determination of their shape, size and morphology as well as the pronuclear characteristics (Kochan et al., 2021 a). The results of these analyses are integral indicators of gamete quality and of the implantation potential of the subsequent embryo. In addition, they predict the frequency of zygote arrest and the occurrence of chromosomal abnormalities (Villagómez and Pinton, 2008; Zamora et al., 2011; Raudsepp and Chowdhary, 2016).

An undoubted advantage of this type of morphological assessment is the non-invasiveness and the possibility of observation using time-lapse systems (Kochan et al., 2022; Kij-Mitka et al., 2022). It is believed that the size of the oocyte or zygote may suggest chromosomal disorders (Mihajlović and FitzHarris, 2018; Bassil et al., 2021), as too large oocytes may be tetraploid. Such oocytes are formed as a result of the lack of cytoplasmic division of oogonia or cytoplasmic fusion of two oogonia (Machtinger et al., 2011). In contrast, the shape of the oocyte, and subsequent zygote, does not have a negative impact on the fertilization process or the quality of the embryo, but often the development of embryos formed from such oocytes/zygotes is delayed (Ebner et al., 2008). The results presented in our study indicate a similar number of blastocysts (P=0.050948) and hatching blastocysts (P= 0.5) developed from regular and irregular shaped presumptive zygotes, despite significant differences in the number of embryos (P= 0.000319) developed from such zygotes. In another study, the average diameters of the oocytes of the domestic cat, Pallas cat, lynx, and serval were all found to be approximately 161±4 μm (Kochan et al., 2021 b). Here, we measured the diameter of the PZ, but assuming no differences between the diameters of oocytes and zygotes, these results should be comparable, and we found an average PZ diameter of 161.37±17.42 μm.

The assessment of the number, size, movement, and morphology of pronuclei in zygotes with light-colored cytoplasm provides information about their potential for further development (https://atlas.eshre.eu/; Athayde Wirka et al., 2014). The presence of pronuclei allows for the determination of fertilization disorders. In addition, an abnormal number of pronuclei (1, 3 or more) in a zygote provides information about abnormal ploidy, which allows the elimination of such embryos from transfer (Diéguez et al., 1995; Mutia et al., 2019). Despite a lot of valuable information provided by the analysis of pronuclei, some authors do not see the benefits of performing this assessment and suggest that it has no relationship with subsequent development (Nicoli et al., 2010; Weitzman et al., 2010). It is also worth emphasizing that in the case of oocytes/zygotes characterized by a dark cytoplasm, pronuclear assessment is limited due to the difficulties, or complete impossibility, of observing internal structures. Due to the inability to see the pronuclei, feline zygotes are referred to as PZs, as confirmation of the correct fertilization is not possible using non-invasive techniques. Only the occurrence of the first embryonic divisions gives certainty about fertilization.

Other assessments based on modified models for human embryo analysis have been attempted, but have also failed to yield the expected results (Kij et al., 2020).

Due to the lack of standards for assessing feline PZ, it is necessary to adapt the evaluation systems used in other species. As we have shown here, in the case of feline PZ, the assessment parameters such as AD1, AD2, AD3, M1, M2 and M3 in the combined form are not related to developmental competence, reaching the blastocyst stage or hatching. It is interesting, however, that the comparison of individual parameters such as AD1 and AD3 with the combined potential to cleave, reach blastocyst stage and hatch, showed a significant relationship, which suggests that the assessment parameters should be treated as separate elements and not considered collectively.

Removal of the zona pellucida of feline embryos produced *in vitro* does not affect their developmental potential and may have a positive effect on embryo quality and viability (Freistedt et al., 2001 b; Veraguas-Davila et al., 2021), however, it may reduce the expression of genes responsible for their implantation capacity (Veraguas-Davila et al., 2021). Our study showed a higher number of presumptive zygotes with a thin zona pellucida that reached the blastocyst stage and started the hatching process. Zona pellucida hardening is a process during which cortical granules are released to prevent polyspermy (Dietzel et al., 2017; Colombo et al., 2023). However, it happens that this process occurs spontaneously already at the IVM stage, often due to inappropriate IVM conditions. As a result, the fertilization rate is reduced (Ducibella et al., 1990; Schiewe et al., 1995; Dadashpour Davachi et al., 2016). A thinner zona pellucida in the presumptive zygotes may prevent or significantly reduce this phenomenon, which in turn may have a positive effect on the developmental competence of such presumptive zygotes. In addition, a higher percentage of blastocysts and hatching blastocysts among presumptive zygotes with a thin zona pellucida is comparable to other studies that showed that the zona thickness was closely related to higher fertilization, implantation, and pregnancy rates (Schmoll et al., 2003; Marco-Jiménez et al., 2012).

Conclusions

The results presented in this study indicate that the complex PZ assessment procedure used for human zy-

gotes is not useful in the case of the cat model. However, single size parameters (PZ diameter, zona pellucida thickness) can be used as predictors of further embryo development. Knowledge of the relationship between measurements of the diameter of presumptive feline zygotes and the thickness of zona pellucida with their developmental potential may in the future be used to develop a new model for the assessment of developmental competences of cat embryos. These studies deserve further investigation to optimize assessment of cat presumptive zygotes.

Conflict of interest

The authors declare no conflict of interest. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

Author contributions

Barbara Kij-Mitka: conceptualization, investigation, formal analysis, funding acquisition, writing original draft, writing review and editing; Julia Gabryś: conceptualization, investigation, formal analysis; Joanna Kochan: supervision, writing review and editing; Agnieszka Nowak: investigation, supervision; Tomasz Szmatoła: formal analysis, data analysis, writing original draft; Sylwia Prochowska: providing research material, methodology, investigation, writing review and editing; Wojciech Niżański: methodology; Monika Bugno-Poniewierska: conceptualization, supervision, writing review and editing.

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