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EVALUATION OF THE DEVELOPMENTAL COMPETENCE OF RABBIT EMBRYOS AT VARIOUS STAGES OF DEVELOPMENT FOLLOWING *IN VIVO* FERTILIZATION*

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The domestic rabbit (Oryctolagus cuniculus f. domesticus) is a valued animal model in experimental embryology because of similar structure and diameter of oocytes and embryos, the bright cytoplasm colour allowing the visualization of pronucleus formation and individual blastomeres, prolificacy and fertility. The aim of the study was to evaluate the developmental competence and viability of rabbit embryos at various stages of development, which were obtained after the post-slaughter flushing from the Fallopian tubes. It has been shown that in vitro embryo culture weakens their development potential. The number of dead blastomeres in the embryos that were not in vitro cultured increased by 9.4% compared to the number of dead blastomeres in the in vitro cultured embryos. In vitro cultured embryos showed a fourfold higher percentage of dead blastomeres (10%) compared to embryos that developed in vivo (2.35%). The present results show that it is difficult to ideally reproduce the conditions of a living organism, and that in vitro cultures demand refinement and optimization.

Key words: rabbit, embryos, embryo competence, fluorochromes, in vitro culture

Domestic rabbit *(Oryctolagus cuniculus* f. *domesticus)* is an animal that is frequently used in biomedical studies (Wierzbicki, 2014), especially in the experimental embryology. Thanks to such features as similar structure and diameter of oocytes and embryos, bright cytoplasm colour allowing the visualization of pronuclei and individual blastomeres (Grygoruk et al., 2013), prolificacy and fertility, it is an excellent model animal applied in order to expand the knowledge on the development of human embryos (Fisher et al., 2012).

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Fertilization in rabbits was described for the first time in 1932 by Gregory Goodwin Pincus, which indicates a great interest in this species (Garcia, 1968). In humans, one of the stages of infertility treatment is *in vitro* fertilization and observation of embryos under laboratory conditions. Difficulties and controversies associated with experimental research on human embryos are associated with the necessity of using model animals in it (Walczak and Bonczar, 2015).

The aim of the study was to evaluate the developmental competence and viability of rabbit embryos at various stages of development, which were obtained after the post-slaughter flushing from the Fallopian tubes.

Material and methods

Preparation of female donors and collection of embryos

Embryos were collected from four sexually mature and healthy females of Popielno white rabbit breed, with body weight from 3.8 to 4.3 kg. Before the planned slaughter, female rabbits were mated several times with males of the same breed. Afterwards, the Fallopian tubes isolated *post mortem* from four females were flushed with physiological solution in order to obtain a suspension from which the embryos were chosen under stereoscopic microscope and then collected. The embryos were collected at different time points from the probable fertilization:

Female rabbit no. 1 – embryos collected one day after the copulation
Female rabbit no. 2 – embryos collected two days after the copulation
Female rabbit no. 3 – embryos collected three days after the copulation
Female rabbit no. 4 – embryos collected four days after the copulation

The embryos were classified based on the morphological structure and the number of blastomeres.

In vitro culture of embryos

The obtained embryos were cultured under the *in vitro* conditions, using TCM 199 medium added with 10% FBS, 16 μg/ml L-glutamine and 4 μg/ml pyruvate under mineral oil in four-well culture plates. The culture was conducted for 24 hours at 38°C with addition of 5% of CO_2.

Fluorescent staining and embryo assessment

In order to visualize the blastomeres with damaged plasmatic membranes, the cells were stained with ethidium bromide that penetrates inside them and gives them red colour. The undamaged plasmatic membranes of blastomeres were stained green using fluorescein diacetate. Following the 24-hour culture, the embryos were placed in a drop of medium on a microscope slide and then stained with the use of fluorochromes: 0.005 mg/ml fluorescein diacetate and 0.05 mg/ml ethidium bromide. Such preparations were assessed under fluorescent

microscope, and blastomeres were classified as live or dead on the basis of the colour of the fluorescent signal.

Results

Twenty embryos were obtained as a result of the procedure of flushing of the Fallopian tubes in four females. All of them were subjected to further observations. Due to the fact that the embryos obtained after the first and the second day following the fertilization were in different developmental stages compared to the embryos obtained after the third and the fourth day following the fertilization – in order to align the developmental stages of embryos from the female no. 1 and the female no. 2, they were *in vitro* cultured (table 1). Embryos were subjected to viability assessment and the number of blastomeres was determined in them (table 2).

Table 1. Developmental competence of rabbit embryos; 2B-two-blastomere embryo; 8B-eight-blastomere embryo

Female	No. of obtained embryos, N (%)	Embryos after *in vivo* fertilization					
		embryo development stage after flushing from female, N (%)			embryo development stage after flushing and *in vitro* culture, N (%)		
		zygote	2B	morula	8B	morula	blastocyst
1	5 (25)	3 (60)	-	-	1 (20)	1 (20)	1 (20)
2	5 (25)	-	4 (80)	-	-	-	4 (80)
3	2 (10)	-	-	1 (50)	-	-	-
4	8 (40)	-	-	5 (62.5)	-	-	-

Discussion of the results

Assisted reproduction with the use of *in vitro* fertilization is becoming one of the most frequently used methods of treatment of infertility in humans (Bensdorp et al., 2016). Studies conducted in model animals, such as rabbits, have provided a lot of valuable information on the quality and the predispositions for further development of embryos (De Los Angeles et al., 2015; Duranthon et al., 2012). In order to properly and appropriately assess this information, such tools and criteria as morphological assessment, development under the *in vitro* conditions (Henrion et al., 1997), cell staining (Amat et al., 2014) and microanalysis of embryo metabolism (Guitart-Mampel et al., 2018; Piliszek et al., 2017) have been used. Morphological assessment using microscope enables to determine the size of blastomeres, degree of their fragmentation as well as cytoplasm colour and granularity (Halacheva et al., 2011; Makarevich et al., 2005).

Table 2. Viability of blastomeres in rabbit embryos

Animal	Embryos	Assessment of embryos		
		no. of blastomeres (N)		
		live	dead (%)	total
Rabbit no. 1	1	-	-	-
	2	-	-	-
	3	8	0 (0%)	8
	4	40	8 (16.6%)	48
	5	23	3 (11.5%)	26
Rabbit no. 2	1	64	8 (11.1%)	72
	2	50	7 (12.3%)	57
	3	64	14 (17.9%)	78
	4	66	1 (1.5%)	67
	5	-	-	-
Rabbit no. 3	1	-	-	-
	2	27	0 (0%)	27
Rabbit no. 4	1	-	-	-
	2	-	-	-
	3	23	0 (0%)	23
	4	-	-	-
	5	27	0 (0%)	27
	6	27	1 (3.6%)	28
	7	17	2 (10.5%)	19
	8	20	0 (0%)	20

Tests performed as part of the present experiment have shown that in vitro embryo culture under laboratory conditions weakens their development potential. The number of dead blastomeres in the embryos that were not *in vitro* cultured increased by 9.4% compared to the number of dead blastomeres in the *in vitro* cultured embryos. Analysis of the quality and velocity of embryo development under laboratory conditions enables to determine the developmental competencies of embryos. One of the main criteria of embryo assessment is its ability to achieve the blastocyst stage, however it is problematic under the *in vitro* conditions (Sugimoto et al., 2015; Sung et al., 2011; Petters and Wells, 1993). Gardner and Schoolcraft (1999) demonstrated that embryo quality may be also assessed based on the number of blastomeres as well as the number of cells in the primitive knot and the trophoblast. This information is obtained among others from the results of using fluorescent staining. Up to now, studies with the use of fluorochromes have been conducted in cattle by Iwasaki et al. (1990), in mice by Mirkes et al. (2001) and in humans by Chatzimeletiou et al. (2005). In the available literature, there is no information on the similar studies carried out in rabbits. Results obtained in the present study have demonstrated that in the later developmental stages of embryos, such as the blastocyst, the percentage of dead blastomeres is higher compared to the embryos composed of several or more than ten blastomeres. The

in vitro cultured embryos showed a fourfold higher percentage of dead blastomeres (10%) compared to embryos that developed *in vivo* (2.35%). The present results show that it is difficult to ideally reproduce the conditions of a living organism, and that *in vitro* cultures demand refinement and optimization.

It seems that the use of rabbits as model animals in embryology is an excellent tool to discover the development of embryos under laboratory conditions. This knowledge can be successfully used in testing and improvement of the procedures applied in fertility treatment clinics in humans, which is related to both the similarities in the development of mammalian embryos and equivalent developmental mechanisms. Such type of studies is extremely important and they should be continued in order to obtain even better results in the treatment of human infertility.

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SUMMARY

The domestic rabbit *(Oryctolagus cuniculus* f. *domesticus)* is a valued animal model in experimental embryology because of similar structure and diameter of oocytes and embryos, the bright cytoplasm colour allowing the visualization of pronucleus formation and individual blastomeres, prolificacy and fertility. The aim of the study was to evaluate the developmental competence and viability of rabbit embryos at various stages of development obtained after *in vivo* fertilization. It has been shown that *in vitro* embryo culture weakens their development potential. Number of dead blastomeres increased by 9.4% in *in vitro* cultured embryos. *In vitro* cultured embryos showed a fourfold higher percentage of dead blastomeres (10%) compared to embryos that developed *in vivo* (2.35%). The present results show that it is difficult to ideally reproduce the conditions of a living organism, and that *in vitro* cultures demand refinement and optimization.

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