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Full Research Article

EFFECT OF METHOD AND MEDIA TYPE FOR *IN VITRO* FERTILIZATION ON EQUINE (*Equus ferus caballus*) EMBRYO DEVELOPMENT

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Abstract

Demonstrating the role of *in vitro* fertilization in the equine family, the current study aimed to monitor the rates of equine *in vitro*-fertilized and cleaved oocytes based on microdroplet and intracytoplasmic sperm injection (ICSI) methods.

The fertilization process was carried out in four main media consisting of Tyrod's albumin lactate pyruvate and TCM-199 (1:1): F (TCM-199); assigned for the ICSI method, FI (contained 0.01 mg/ml heparin + 0.01 ng/ml hypotaurine), FII (contained 0.3 mg/ml calcium chloride + 0.1 mg/ml magnesium chloride) and FIII (contained 0.05 mg/ml hypotaurine-epinephrine).

The results indicated an increase in the fertilization rate by the ICSI method (65.71%) compared to the microdroplet method (39.25%) (p 0.04). The rates of unfertilized and degenerated oocytes in the ICSI method decreased, compared to the microdroplet method, to 8.57% and 25.72%, respectively; p< 0.001. The rate of fertilized oocytes in the FI medium increased to 47.61% (p< 0.05) compared to oocytes of FII and FIII treatments (37.64% and 30.00%, respectively). On *in vitro* culture, the rate of cleavage increased to 74.62% in the CI medium (SOF) compared to those in the CII medium (DMEM-F12) (61.29%); p <0.001. Zygotes cultured in the CII medium achieved a higher rate of blastocyst formation (30%) compared to those in the CI medium

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(26.31%); p< 0.001. Applying the ICSI method and SOF culture media led to high yields of equine embryos.

Key Words: cleavage, culture media, equine, *in vitro* fertilization, oocytes

INTRODUCTION

Assisted reproductive technologies (ART) have revolutionized animal breeding by providing accelerated genetic improvement programs through technologies such as estrus synchronization, ovulation induction, and embryotransfer (ET). These biotechnologies have increased the importance of females in the genetic improvement process, and through ART, both male and female genetics can be harnessed to produce elite offspring with unique genetic structures. This has been particularly successful in goats, sheep, and horses (*Equus ferus caballus*), where there productive physiology and economic value of these animals make them well-suited for ART. Furthermore, these technologies not only improve reproductive efficiency but also address challenges in animal reproduction.

Although the use of ART in animal breeding is still in its early stages, it provides a promising approach for accelerating genetic improvement programs in various farm animals (Ribeiro et al., 2016; Silva et al., 2016). The development of ART has been linked to a variety of basic factors, such as those related to the animal's external environment (e.g., nutrition, care, and reproductive efficiency), and factors related to *in vitro* embryo production (IVEP) (e.g., conditions of maturation, fertilization, and culture) (Gallegos et al., 2022). Oocyte collection is a crucial step in the production of high-quality embryos for use in ART in farm animals.

The selection of suitable follicles for oocyte collection is important to ensure the quality and developmental potential of the oocytes. The follicles used in ART can be categorized into three types: small, medium, and large. Small follicles are typically less than 3 mm in diameter and contain immature oocytes, while medium follicles range from 3 to 6 mm in diameter and contain both immature and mature oocytes. Large follicles are typically greater than 6 mm in diameter and contain mature oocytes that are ready for ovulation. The selection of the most appropriate follicles for oocytes collection can have a significant impact on the success of ART in farm animals (Rizos et al., 2005; Singh et al., 2020).

Compared to *in vitro* maturation (IVM) and *in vitro* culture (IVC), *in vitro* fertilization (IVF) was characterized by a unique development to obtain an ample yield of embryos. In general, the current studies tended to apply the intracytoplasmic sperm injection method (ICSI), as it is a more effective method in ensuring the fertilization of oocytes compared to the previous method, which is metaphorically defined as IVF (microdroplet method) (da Silva et al., 2020). Moreover, the ICSI method is effective in overcoming a large number of male-related obstacles (e.g., semen characteristics, sperm concentration and the deteriorating morphological characteristics of sperm) (Glenn et al., 2021).

IVEP of equines starting from immature oocytes provides an important alternative based on collecting oocytes from slaughtered or live mares. In addition, IVEP produces a source of oocytes or embryos at advanced stages of development. The Lipizzaner breed is one of the oldest European breeds, dating from the 16th century in a specialized Spanish riding school that used only this breed (Canesin et al., 2017). The breed first originated in a village called Lipica, which is located in Slovenia. In Romania, due to the strategic importance of this breed, used as a racehorse or as an important meat source, efforts have been directed to preserve the genetic purity and structures of this breed and keep them in gene banks. Hence, this study aims to determine the effect of two IVF techniques and two culture media on IVF and cleavage rates of the Lipizzaner breed horse. The study is also implicitly aimed at improving the techniques for preparing sperm and oocytes used in IVF and at examining the methods and solutions used in culturing complimentary embryos.

MATERIALS AND METHODS

The research was carried out in the Laboratory of Biotechnologies of the Reproduction, Obstetrics and Gynecology Department of the Faculty of Veterinary Medicine Cluj-Napoca, Romania. Tissue samples were collected after slaughter, which was performed according to authorized practices, and there was no need for experimental approval to protect animal welfare.

Reagents. Unless otherwise mentioned, all chemicals used in the experiments were taken from SIGMA CC (St Louis, MO, USA).

Oocyte collection. Ovaries were collected after 10 minutes from slaughtered Lipizzaner mares *(Equus ferus caballus)* and transported to the laboratory in a solution of sodium chloride 0.9% supplemented with streptomycin (100 μm/ml) and penicillin (100 IU/ml) or in a solution of phosphate buffer saline (PBS) supplemented with the antibiotics, at a temperature of 38 ºC to ensure the viability of oocytes was maintained until processing. Recovery of oocytes was achieved by using two methods, aspiration and ovarian scraping. The aspiration method was typically performed by puncturing the follicle containing the oocytes with a fine needle (20 G), and aspirating (suctioning) the contents of the follicle, including the oocyte (Gasparrini et al., 2011). The procedure of ovarian scraping involved using a small scraper to create tiny punctures in the ovarian tissue, which could help to release the highest possible yield of oocytes (Merton et al., 2000). The total number of oocytes obtained was 930 oocytes from 376 mares aged between 3 and 18 years during the reproduction season. Altogether, 633 oocytes (68.06%), on average 1.86 oocytes/mare, were obtained by the aspiration method, and 297 oocytes (31.94%), on average 0.78 oocytes/mare, were obtained by the ovarian scraping method. The collected oocytes were examined using an inverted microscope (Nikon Eclipse TS100, inverted phase contrast, Nikon objectives) at 20x magnification and were evaluated morphologically. Oocytes were selected based on completed growth and having more than four layers of cumulus cells with homogeneity

in the cytoplasm. A total of 645 oocytes were classified as cultivable, i.e., 462 oocytes (72.98%) by aspiration and 183 oocytes (61.61%) by ovarian scraping and so were used for *in vitro* maturation (Zare et al., 2021).

Experimental design. According to Montgomery (2020), the current experimental study employs a one-factor random design to investigate the effects of various factors on multiple characteristics of embryo development. Specifically, the study examines the impact of the method of fertilization and the type of media used in fertilization and culture on fertilization rates, cleavage rates, degeneration rates, and embryo stage. The factors of interest, i.e., the method of fertilization and type of media, were randomly assigned to different experimental conditions while keeping all other variables constant to identify the causal relationships between the manipulated factors and the observed outcomes, providing valuable insights into the complex processes involved in embryo development. Using Tyrode's albumin lactate pyruvate medium (TALP) as the only primary medium for the microdroplet method, three fertilization media were prepared (FI, FII, and FIII). The F medium was specified to the ICSI method, as shown in Table 1. To follow up on the subsequent division processes of early embryos, two culture media were used for *in vitro* culture (IVC) of zygotes, as shown in Table 2.

Table 1. Fertilization media composition used in in vitro fertilization of equine (*Equus ferus caballus*) oocytes.

Medium		Product	Proportion
ICSI method	F	TCM 199 buffered with Hepes	10 ml
		BSA	1 mg/ml
Microdroplet method	FI	TALP	10 ml
		Heparin	0.01 mg/ml
		Penicillin	0.06 mg/ml
		Hypotaurine	0.01 ng/ml
	FII	TALP	10 ml
		Calcium chloride	0.3 mg/ml
		Magnesium chloride	0.1 mg/ml
	FIII	TALP	10 ml
		Penicillin-hypotaurine- epinephrin (PHE)	0.05 mg/ml
		BSA (Fraction V)	0.04 µl

TCM 199:tissue culture medium-199
BSA: Bovine Serum Albumin
TALP: Tyrode's albumin lactate pyruvate medium

***In vitro* maturation.** The collected oocytes were subjected to three washes with hepes-buffered tissue culture media (TCM) followed by an additional wash with TCM. Oocytes (10 to 15) in cumulus oophorous complexes (COCs) were transferred to 4-well Petri dishes containing the same medium and placed under sterile silicone oil.

The dishes were then incubated in a 5% CO_2, 90% N_2, and 5% O_2 atmosphere at 38.5 ºC for 27 hours. For the IVM process, 25 mm hepes-buffered TCM-199 supplemented with 2 mL sodium pyruvate, 1 mm l-glutamine, penicillin (75 mg/ml), streptomycin (50 mg/mL), and 10% fetal calf serum (FCS) of the total volume was used. After 28 hours of IVM, the COCs were examined under an inverted microscope to detect the formation of the first polar body (Pallottino et al., 2015).

Table 2. Culture media composition used for *in vitro* culture of equine (*Equus ferus caballus*) oocytes

Medium	Product	Proportion
CI	SOF	10 ml
	Sodium pyruvate	0.33 mM/l
	Lactate acid	3.3 mM/l
	BSA	4 mg/l
	Glutamine	200 mM/l
CII	DMEM-F12	10 ml
	FCS	10%
	Penicillin--streptomycin powder	0.1 mg/ml

SOF: synthetic oviductal fluid
DMEM-F12: Dulbecco's Modified Eagle Medium and Ham's F-12
TALP: Tyrode's albumin lactate pyruvate medium

Semen preparation, sperm capacitation, *in vitro* fertilization and intracytoplasmic sperm injection methods. The seminal material used in the IVF protocol of oocytes required prior training in estimating sperm capacity and the ability to penetrate and fertilize them. Vitrified semen straws of proven stallions of the same breed were used. To cap the sperm, semen straws were thawed by placing them in warm water at 37 ºC for 40 seconds. The straw contents were placed in a 12 x 55 mm conical tube with a lid. The sperm preparation was made using the swimming-up method described by Choi et al. (2016). Briefly, 2 ml of standardized medium (sperm TALP medium, which had previously been equilibrated by incubation at 39 °C and 5% CO_2 for 2 hours) was used. Next, the same amount of defrosted material was added. The resulting solution was homogenized and centrifuged for 7 minutes at 363 rpm in order to sediment live sperm in the shape of a button in the basal part of the tube. The sediment was resuspended in 800 µl TALP medium. Prepared tubes were incubated for 1 hour at 37-38 ºC to collect the spermatozoa. Finally, 400 µl of the supernatant in which the motile sperm were collected by laboratory examination of the motility and viability of the sperms, as well as the concentration of sperm/ml (Parrish, 2014). The post-thaw motility objectively was tested by computer assisted sperm analysis (CASA). In CASA system, a progressively motile sperm is defined as one that has an average path velocity (VAP) of > 50 microns/second and is moving with a straightness value (STR) of > 75%. In the microdroplet method, drops of 35 µl of *in vitro* fertilization medium (FI, FII, FIII) were placed in four well Petri plates with a diameter of 60 mm. The medium

drops were covered with mineral oil and were incubated at 38.2 ºC, 100% humidity, 5% CO_2, 2 to 3 hours for equilibration. Mature COCs were completely denuded of cumulus cells and were added to medium drops (10 to 15 oocytes/drop). Then, 7 µl of sperm (concentration of 2 x 10^6 sperms/mL) was added to each drop. The plates were incubated at 38.2-38.5 ºC, 5% CO_2, 100% humidity, for 24 hours (Table 1). Following the IVF period, the Petri dishes were examined under the stereomicroscope and inverted microscope for the detection of pronuclei (zygotes)**.** Microinjection was performed based on previous paper (Choi et al., 2016) with some modifications to fit the experimental environment. Sperm injection was performed under the strict control of the inverted microscope (Olympus Microscope lx51, inverted fluorescence and phase contrast) and the micromanipulator. Suspension of spermatozoa diluted with 5% PVP (polyvinylpyrrolidone) in 0.9% saline solution was prepared and disposed of in three drops, 2 round and one elongated. The first drop (10 µl polyvinylpyrrolidone saline) was used to wash the pipette. The second one (10 µl) comprised the sperm suspension in polyvinylpyrrolidone saline. The third elongated drop (150 µl) contained TCM-199 buffered with Hepes and 1mg/ml BSA. After covering the droplets with mineral oil, six denuded oocytes were placed inside each third drop. A sperm was taken from the sperm-containing drop by aspiration of the tail and injected into the oocytes. The fixation of the oocyte with the pipette was done according to the position of the polar body and corresponded to the 12 or 6 o'clock positions on a clock face. This arrangement avoids the destruction of the metaphase spindle by the deep insertion of the pipette. Following injection, the sperm pipette was inserted into the ooplasm at the 3 o'clock position, and moderate pressure was applied to break the oolemma. A small amount of ooplasm was aspirated into the injection pipette. Aspirated ooplasm and sperm were then injected into the ooplasm in as small volume as possible. The pipette was easily extracted. In the fertilization protocols, the evaluation of oocytes was done based on the morphological examination completed with microphotometric evaluation.

***In vitro* culture.** The culture process of resulting zygotes was carried out partly based on a method in a previous paper by Sovernigo et al. (2017). Briefly, at the end of IVF, presumptive zygotes were washed in three successive solutions assigned for culture (CI and CII, based on SOF and DMEM-F12, respectively), vortexed and transferred to the specified culture media (Table 2). Zygotes (3 to 4) were placed in each well and incubated for 30 hours under the same conditions of IVM. Cleavage rates were recorded during days 6 and 7 post culture. The stage of embryo development during the culture period was established according to the size of the embryo, appearance, number of blastomeres, compaction of internal mass, the occupancy of the perivitelline space and the presence of blastocoel cavitation. The embryonic stage was determined based on the continuity of division or arrest.

Statistical analysis. The data, including the factors (method of fertilization, type of fertilization medium and type of culture medium) and the studied characteristics (IVM, IVF, cleavage, degeneration of oocytes and embryo stage) were analyzed by

Chi-Square with the contingency table method according to the log-linear model. To investigate the differences across the resulting rates, Fisher's exact test was applied at the specified significant levels ($p<0.05$). In our statistical analysis, we relied on the 14.3 version of statistical analysis package (SAS) (SAS, 2017).

RESULTS

In vitro fertilization (IVF). In the microdroplet method (Fig1), a significant difference was noticed in fertilization rates ($p<0.05$) which ranged between 30% and 47.61% (Table 3). In this context, oocytes fertilized in the FI medium achieved the highest rates (47.61%), followed by oocytes fertilized in FII and FIII media (37.64% and 30%, respectively). Figure 2 A shows the moment of penetration of the sperm into the zona pellucida membrane. In fertilization environments, unfertilized oocytes were without sperm attached or penetrated through the zona pellucida membrane. The degenerated oocytes were characterized by deterioration in the cytoplasm and granulation (Figs 3 and 4). Furthermore, no significant differences were observed in the rates of unfertilized or degenerated oocytes. The lowest rate of unfertilized oocytes was recorded in the FI medium (21.90%), whereas in the FII and FIII media, the rates were 30.58% and 27.5%, respectively. The rate of degenerated oocytes following fertilization ranged between 31.76% (FII medium) and 42.5% (FIII medium).

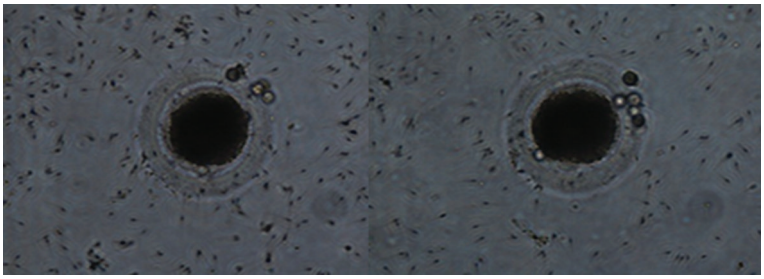

Figure 1. Fertilized mare oocytes (*Equus ferus caballus*)

Table 3. Rates of IVF, non-fertilized and degenerated equine (*Equus ferus caballus*) oocytes across three fertilization media

IVF-Medium	Incubated oocytes	Fertilized		Non-fertilized		Degenerated	
	No.	No.	%	No.	%	No.	%
F I	105	50	47.61 ^{a}	21	20.00	34	32.38
F II	85	32	37.64 ^{ab}	26	30.58	27	31.76
FIII	80	24	30.00^{b}	22	27.5	34	42.50
P		<0.05		NS		NS	

NS: not significant. ^{a,b} – Values in the same column with different lowercase characters are statistically significant at $p<0.05$.
F I: 10 ml TALP, 0.01 mg/ml heparin, 0.06 mg/ml penicillin and 0.01 ng/ml hypotaurine.
F II: 10 ml TALP, 0.3 mg/ml $CaCl_2$ and 0.1 mg/ml $MgCl_2$.
F III: 10 ml TALP, 0.05 mg/ml penicillin-hypotaurine- epinephrine and 0.04 µl BSA.

In the ICSI method (Figure 2 B), we used 35 oocytes based on morphological examination completed with morphometric examination. The structural elements followed in assessing the degree of maturation were: oocytes with a diameter greater than 110 μm, compaction and expansion of the cumulus cells layer at a size greater than 40 μm, integral pellicle membrane with a minimum thickness of 13 μm, homogeneous cytoplasm, granulated and uniformed perivitelline space. The rates of fertilized, unfertilized and degenerated oocytes were 65.71%, 8.57% and 25.72%, respectively (Table 4).

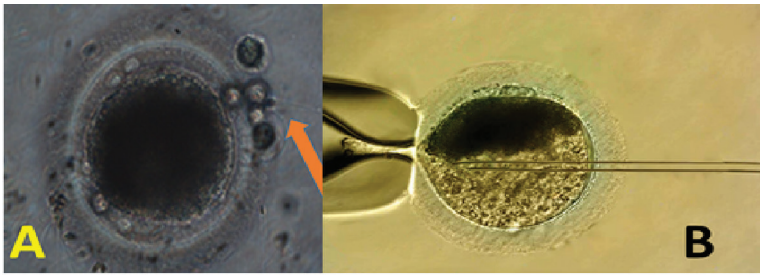

Figure 2. Fertilization of equine oocytes (*Equus ferus caballus*): **A:** penetration of the sperm attached to zona pellucida membrane. **B:** fertilization of oocytes by the ICSI method

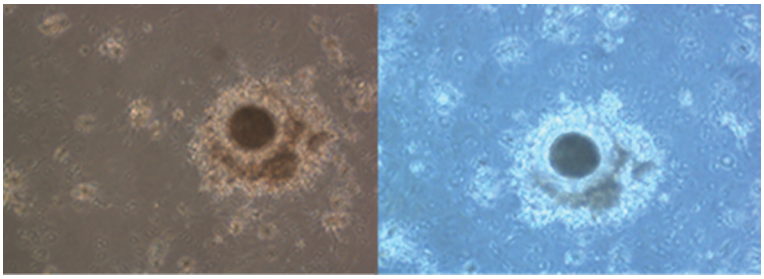

Figure 3. Non-fertilized oocytes (*Equus ferus caballus*)

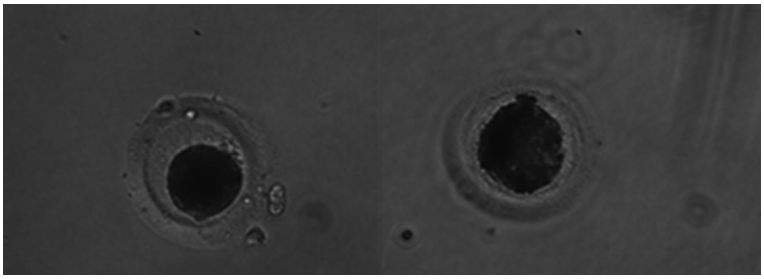

Figure 4. Degenerated mature oocytes following fertilization (*Equus ferus caballus*)

Table 4. General rates of IVF, non-fertilized, and degenerated equine *(Equus ferus caballus)* oocytes in the two methods of IVF across four media

Medium	Oocytes	Fertilized oocytes		Non-fertilized oocytes		Degenerated Oocytes	
	No.	No.	%	No.	%	No.	%
F (ICSI method)	35	23	65.71[a]	3	8.57[a]	9	25.72 [a]
FI+FII+FIII (microdroplet method)	270	106	39.25[b]	69	25.55[b]	95	35.18[b]
P		<0.04		<0.001		<0.001	

[a,b] – Values in the same column with different lowercase characters are statistically significant at $p<0.05$

F I: 10 ml TALP, 0.01 mg/ml heparin, 0.06 mg/ml penicillin and 0.01 ng/ml hypotaurine.

F II: 10 ml TALP, 0.3 mg/ml $CaCl_2$ and 0.1 mg/ml $MgCl_2$.

F III: 10 ml TALP, 0.05 mg/ml penicillin-hypotaurine- epinephrine and 0.04 µl BSA.

On comparing the general rates of both methods, it was noticeable that the rates of oocytes fertilized by the ICSI method increased, with a difference of 26.46% ($p<0.04$), compared to oocytes fertilized by the microdroplet method. The rates of unfertilized and degenerated oocytes increased when the microdroplet method was used ($p<0.001$), reaching a difference of 16.98% and 9.46%, respectively, compared to their counterparts in the ICSI method.

Cleavage and embryo stage. Table 5 shows a significant difference ($p<0.001$) in the cleavage and degeneration rates of embryos cultured in the two different media (CI and CII) across the cleavage and embryo stages (Figure 5). The cleavage rate was higher (74.62%) and the degeneration rate was lower (25.38%) in embryos cultured in the CII medium compared to those in the CI medium (61.29% and 13.78%, respectively). Additionally, Table 6 reveals the blastocyst rate increased ($P<0.001$) in embryos cultured in the CII medium (30%) compared that in the CI medium (26.31%). Conversely, the rates of arrest at the blastomere (2-8 cells) and morula stages in the CI medium were

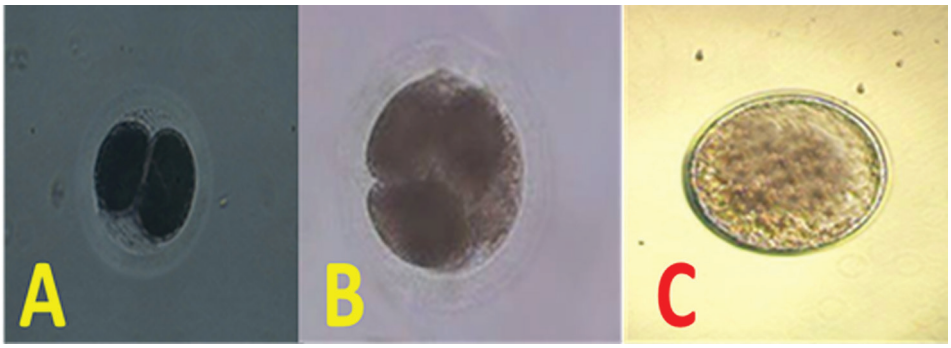

Figure 5. Different stages of equine (*Equus ferus caballus*) early embryos: **A**: 2 cells. **B:** 4 cells. **C:** early blastocyst

28.95% and 44.74%, respectively, compared to 26% and 44%, respectively, in the CII medium.

Table 5. Rates of development and degeneration of equine *(Equus ferus caballus)* early embryos cultured in two different media

Culture medium	Fertilized oocytes	Developed embryos		Degenerated embryos	
CI	No.	No.	%	No.	%
	62	38	61.29[a]	24	38.71[a]
* CII	67	50	74.62[b]	17	25.38[b]
P		<0.001		<0.001	

[a,b] – Values in the same column with different lowercase characters are statistically significant at $p<0.05$
C1: 10 ml SOF, 0.33 mM/l sodium pyruvate, 3.3 mM/l lactate acid, 4 mg/l BSA and 200 mM/l Glutamine.
C2: 10 ml DMEM-F12, 10% FCS and 0.1 mg/ml Pen Strep powder.

Table 6. Rates of 2-8 cell, morula and blastocyst stages of equine early embryos *(Equus ferus caballus)* cultured in two different media

Culture medium	Developed embryos	Embryonic stage					
		2-8 cell		Morula		Blastocyst	
		No.	%	No.	%	No.	%
CI	38	11	28.95[a]	17	44.74[a]	10	26.31[a]
CII	50	13	26.00[b]	22	44.00[b]	15	30.00[b]
P		<0.001		<0.001		<0.001	

[a,b] – Values in the same column with different lowercase characters are statistically significant at $p<0.05$
C1: 10 ml SOF, 0.33 mM/l sodium pyruvate, 3.3 mM/l lactate acid, 4 mg/l BSA and 200 mM/l Glutamine.
C2: 10 ml DMEM-F12, 10% FCS and 0.1 mg/ml Pen Strep powder.

DISCUSSION

Compared to ruminants, the application of ART to horse gametes was delayed. Perhaps this delay may be due to the strategic economic importance of horses as animals that are scarce in number and are not used for meat production except in very few countries. Accordingly, obtaining the ovaries of mares from slaughterhouses is extremely difficult due to the keen interest of breeders in horses and not sending them to slaughter. Subsequently, the processes of IVM and IFV by various methods were initiated. Altering IVF conditions can significantly affect the developmental competence of the oocyte as reflected in the high yields of the morula and blastocyst (Liu et al., 2015; Herrick et al., 2020).

Regardless of the method of fertilization, the results obtained in our study (Tables 3, 4, and 5) can be attributed to a group of interrelated factors. More specifically, the increased secretion of estradiol from the pre-ovulatory follicle is caused by episodic pulses of increased frequency of luteinizing hormone (LH) that stimulate aromatase activity and then significantly inhibit it. Thus, the last stages of follicle development before ovulation are determined by the pattern of LH secretion (Lawrenz et al., 2020). In addition, in the dynamic follicular wave context, hormonal behavior in the ovary influences the development of follicles according to a specific synchronization between activated growth factors (e.g., epidermal growth factor, insulin-like growth factor (IGF) 1 and IGF2) and inhibitory factors (e.g., inhibin A and inhibin B) to finally result in the determination of the dominant follicle (Raju et al., 2013; Lopera-Vasquez et al., 2017; Rua et al., 2019; Lawrenz et al., 2020). Above all, the vital activities occurring in the ovarian surface epithelium tissues play an important role in the ovarian activity during the reproductive period (Radovanović et al., 2018). Therefore, the ovaries collected in slaughterhouses contain oocytes of different sizes. As a result, the success of fertilization is determined mainly by the developmental competence of the collected oocytes, which in turn is related to the diameter of the oocyte.

Besides, semen quality (fresh or chilled), sperm viability and DNA integrity play a fundamental role in determining fertilization rates (Morotti et al., 2014; Martinez-Rodriguez et al., 2020; Androni et al., 2021; Hyde et al., 2022; Felix et al., 2022). By comparing the results of fertilization methods (Table 4), it was clear that the rates of oocytes fertilized by the ICSI technique were high. Significantly, most of the relevant studies indicated that fertilization of oocytes by the ICSI technique greatly improves the yield of fertilized oocytes, especially since it is the best method for injecting frozen sperm. Our results coincided with most studies; Hendriks et al. (2021) documented higher fertilization abnormalities in sheep oocytes following ICSI compared to conventional fertilization. However, healthy lambs were born, but blastocyst rates were low. The study of Dell'Aquila et al. (2015) showed that equine oocytes with expanded cumulus cells produced higher IVF rates ($P < 0.01$) when fertilized by ICSI compared to the common method (microdroplet) (52.2% vs 17.1%). Moreover, oocytes with compact cumulus cells achieved low fertilization rates (14.7% vs 4.4%). In the microdroplet method in our study (Table 3), the fertilization medium affected the IVF rates. Perhaps the complex relationship between IVM and IVF media significantly influences the outcomes of these two stages. Many studies used many media in IVM and IVF stages, and results have been obtained in a wide range. Bertero et al. (2017) and Grøndahl et al. (2018) used five IVM media and TALP medium (for IVF). According to the study, the rates of the two-cell embryos ranged between 76% and 82%, morula and blastocyst rates ranged between 25% and 32%, while the rates of blastocyst ranged between 12% and 19%. The use of Waymouth's medium and Ham's F-12 for IVM led to a decrease in the rates of two-cell embryos (52% and 37%, respectively). In another study, within the same two culture media, the morula and blastocyst rates were 27% and 9%, respectively. However, the blastocyst rates were 6%

and 3%, respectively (De Munck et al., 2013). In the current study, in IVM and IVF, it was found that the CII medium was the best in terms of cleavage rates and embryo viability (Tables 5 and 6). Both the current study and many previous studies have demonstrated the effects of different culture media in the cleavage stage of different species. For instance, the blastocyst rates resulting from injecting sheep oocytes with two sperm protocols (freeze- dried and frozen-thawed spermatozoa) were 10% and 19%, while it was 33% in the IVF method (Ressaissi et al., 2021). The study of Hajian et al (2022) showed that the utilization of the commercial bracket-oliphant (BO) medium resulted in a significantly greater blastocyst rate ($p<0.05$) compared to the SOF medium. To note, compared to ruminants, the equine blastocyst can be obtained by initial culture in a modified SOF, followed by transfer to DMEM/ F-12 medium at day 5 approximately. The blastomeres can develop into blastocysts starting from day 7 post culture; this requires a medium degree of expansion and the formation of an organized outer layer of trophoblast cells (Jacobson et al., 2010). Unlike in ruminants, the expansion of the blastocoel with a thin trophoblast layer and the formation of an inner cell mass do not occur in the equine cell line. However, despite the studies conducted in this regard, little work has been done on the equine line compared to other species (Carnevale, 2016).

CONCLUSION

According to the results obtained in the current study, by using 10 ml of TCM 199 buffered with hepes and 1 mg BSA, it was found that the ICSI method for treating horse oocytes improved the yield of early embryos. In addition, culturing the zygotes in a culture medium containing 10 ml DMEM-F12, 10% FCS, and 0.1 mg/ml penicillin-streptomycin powder contributed to supporting the fertilized oocytes to obtain high blastocyst rates.

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Authors' contributions

H. A. and O.M. conceived and planned the experiments. H.A., O.M., and I.G. carried out the experiments. H.A., O.M. planned and carried out the simulations. H.A. and O.M. contributed to sample preparation. H.A., O.M., I.G., and L.B. contributed to the interpretation of the results. H.A. and O.M. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Competing interests

The authors declare that they have no competing interests pertinent to the present study.

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UTICAJ METODA I VRSTA MEDIJUMAKOD *IN VITRO* FERTILIZCIJE I RAZVOJA EMBRIONA KONJA *(Equus caballus)*

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Kratak sadržaj

Zbog sve većeg značaja *in vitro* fertilizacije kod konja, studija je imala za cilj da prati uspešnost *in vitro* oplođenih oocita na osnovu "microdroplet" i ICSI metoda. Proces *in vitro* fertilizacije sproveden je u četiri glavna medijuma koji se sastoje od Tirodovog albumin laktat piruvata i TCM -199 (1:1): F (TCM -199); dodeljen za ICSI metodu, FI (sadržao 0,01 mg/ml heparina + 0,01 ng/ml hipotaurina), FII (sadržao 0,3 mg/ml kalcijumhlorida + 0,1 mg/ml magnezijumhlorida) i FIII (sadržao 0,05 mg/ml hipotaurin- epinefrina). Rezultati su ukazali na povećanje procenta oplodnje ICSI metodom (65,71%) u odnosu na "microdroplet" metodu (39,25%) (p 0,04). Procenti neoplođenih i degenerisanih oocita u ICSI metodi su smanjeni na 8,57% i 25,72% respektivno; p< 0,001. Procenat oplođenih jajnih ćelija u medijumu FI je veći (47,61%, p<0,05) u poređenju sa oocitima tretiranim FII i FIII (37,64% i 30%, respektivno). Procenat razvoja oocita je veći (74,62%) u CI medijumu (SOF) u poređenju sa CII medijumom (DMEM-F12) (61,29%); p <0,001. Zigoti kultivisani u medijumu CII su u najvećem procentu dostigli formu blastociste (30%) u poređenju sa onima u medijumu CI (26,31%); p< 0,001. Primena ICSI metode i SOF medija dovela je do dobijanja velikog broja konjskih embriona.

Ključne reči: ovum *pick-up*, medijumi za embrione, *in vitro* fertilizacija, konji, oociti