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Morphocytometric Evaluation of Equine Oocytes Following *In Vitro* Maturation

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RESEARCH ARTICLE

Abstract

In the current study, 930 equine oocytes were collected, 765 (82.25%) of which were considered viable for *in vitro* maturation. All selected oocytes were matured for 27 h at 38.50 ^{0}C in an atmosphere of 5% CO_2 in humidified air. 25 mm hepes-buffered TCM-199 supplemented with 2 mm sodium pyruvate, 1mm l-glutamine, penicillin (75 mg/ml), streptomycin (50 mg/ml) and 10% fetal calf serum was used for *in vitro* maturation. Following maturation, matured COCs were examined to investigate for first polar body formation. The morphocytometric assessment was performed using the motic image plus (MIP) software with an inverted microscope. Morphocytometric examination results showed highly significant differences ($p<0.001$) among groups of matured oocytes (zona pellucida thickness; <13 µm, cumulus oophorus thickness; < 10 µm and oocytes diameter; < 100 µm), where the rates were 63.39%, 33.59% and 30.58%, respectively. The results of morphocytometric evaluation based on zona pellucida thickness, cumulus oophorus thickness and oocyte diameter of the total 765 cultured oocytes showed that 197 oocytes (25.75%) were classified as excellent mature, 108 oocytes (14.11%) as mature good, 203 oocytes (26.53%) as immature and 257 oocytes (33.59%) were considered as degenerated. It is concluded thatthe oocytes rates differed according to the parameters (cumulus oophorus, oocytes diameter, zona pellucida).

Keywords: Equine; oocyte culture**;** oocyte diameter; maturation; morphocytometric assessment.

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INTRODUCTION

The development of assisted reproductive technologies (ART) in the horse dates back to the late nineteenth century when Walter Heape established the first equine pregnancies obtained by artificial insemination (Heape, 1898). Since that time, progress in assisted reproduction in the horse has been continuous although at an irregular pace compared with other domestic species (Squires, 2005). Oocyte morphological evaluation is the most often intimidating moment of the technique. This step is required because of unexplained use of degenerated oocytes in fertilization without chance of development (Krisher, 2004; Assumpsao et al., 2002). The success of morphological assessment is based on three main elements: training, experience and equipment working properly. According to some experiments, studies have reported that the classification accuracy is up to 95% for oocytes collected and blastomeres when using a stereo microscope (10x to 40x) (Valenzuela et al., 2017). Oocyte quality has impacts on embryonic survival, establishment and maintenance of pregnancy and fetal development. Assisted reproductive technologies which involve the collection of

immature oocytes followed by in vitro maturation, disrupt this process resulting in a reduction in oocyte quality (Carnevale, 2016). Oocyte ability indicators to be fertilized are represented by the presence of layers of compact cumulus cells around the oocyte and homogeneous cytoplasm (Chan et al., 2009). Oocytes evaluation needs to identify the correct morphological changes, the best procedure for oocytes classification consists of isolation and removing the debris by repeated washings (3times) and separating into groups of quality (Hinrichs, 2010b). Classical methods of classification based on morphological classification proved over time that they were insufficient and subjective. The selection of oocytes that did not complete nuclear and cytoplasmic maturation, led to a small percentage of viable embryos (Morris, 2018). A realistic alternative that does not affect the integrity of oocytes morphology and offers the possible selection and rigorous sorting is based on the introduction of morphometric assessments in practice to establish the structural integrity of oocytes (Ruggeri et al., 2015). According to the nature and directions of laboratory work in the production of embryos, the process of collecting eggs in slaughterhouses is one of the most important procedures because it determines to a large extent the feasibility of laboratory production of embryos. It was necessary to pay attention to the classification of oocytes descended mainly from follicles of varying size and developmental degree, as well as the state of the dynamic follicular waves and what they determine from the dominant follicle. Here, this paper aims to improve the system of morphological assessment of equine oocytes by introducing the current practice to select them for in vitro fertilization. These assessments allow stabilisation of the morphological and morphometric parameters after maturation. We also aimed to draw up a classification of oocytes based on the evaluated morphometric criteria, thus establishing suitability for in vitro fertilization.

MATERIALS AND METHODS

Animals and ovaries collection

The research was conducted in the Reproductive Biotechnology Laboratories of the Faculty of Veterinary Medicine, USAMV Cluj-Napoca. Ovaries were collected after 10 minutes from slaughtered mares and transported to the laboratory in a solution containing 0.9% sodium chloride supplemented with 100 µm/ml streptomycin and 100 i.u/ml penicillin or in a solution of phosphate buffer saline (PBS) supplemented with antibiotics at a temperature of 30-33 ºC to ensure maintaining the viability of oocytes until processing.

Oocytes collection

Recovery of oocytes was achieved by using both methods of aspiration and scraping. 930 equine oocytes were collected, examined by inverted microscope at 10-20x and evaluated morphologically. Only those that had compact, three or more complete layers of cumulus cells and fully grown oocytes with homogenous cytoplasm were selected for the experiment. 765 of 930 oocytes (82.25%) were classified as cultivable and used for *in vitro* maturation (Fathi and Elkarmot, 2021).

In vitro maturation procedures

All selected oocytes were washed three times in TCM-199 culture medium and once in maturation medium. Next, the oocytes were placed in four-well dishes (10-15 oocytes/well) under sterile silicone oil and matured for 27 h at 38.5 °C in an atmosphere of 5% CO_2 in humidified air. The maturation medium consisted of 25 mm hepes-buffered TCM-199 supplemented with 2 mm sodium pyruvate, 1mm l-glutamine, penicillin (75 mg/ml), streptomycin (50 mg/ml) and 10% fetal calf serum. Following the maturation period (27 hours), the oocytes were examined microscopically to investigate the first polar body as a key indicator of the maturation process (Mohamed et al., 2018).

Experimental design

According to the studied parameters (zona pellucida,cumulus oophorus and oocyte diameter), the oocytes were distributed randomly to a group of classes within each parameter as shown in Table 1.

Table 1. Classes of studied parameters of equine oocytes subjected to the study

Parameter	Thickness (µm)
Zona pellucida	< 10
	10-12
	>13
Cumulus oophorus	< 10
	10-40
	40-60
	>60

Parameter	Thickness (µm)
Oocyte diameter	< 100
	100-120
	120-130
	>130

Morphological Examination

In vitro culture was performed for a total of 765 oocytes which were classified as cultivable. After completion of the culture period, the evaluation of matured oocytes was determined by general examination and morphological evaluation using an inverted microscope (Ciupe et al., 2005). Next, the oocytes were classified based on the morphology into two basic categories (mature, degenerated) depending on cumulus expansion, the appearance of the first polar body, the size of perivitelline space, the integrity of the zona pellucida and cytoplasm appearance (Brum et al., 2005).

Morphometric Examination

We made a new assignment regarding oocytes quality classification post examination and morphometric measurements. Morphocytometric analysis was performed using the MIP software through an inverted microscope (Bertero et al., 2017), taking into study the thickness of the zona pellucida and cumulus expansion after maturation, oocyte diameter, presence and size of the first polar body. Morphocytometric measurements were performed and complemented by morphological examination; we allowed the reinstatement of oocytes into four quality classes according to oocyte diameter, expanded cumulus size, pellucida membrane thickness and the length and width of the first polar body (Claes et al.,2016).

Statistical Analysis

Pearson's Chi-square of contingency tables and the exact Fisher test were used to analyzing the data relating to the targeted oocytes (oocyte viability, meiotic maturation, cumulus cell membrane integrity, oocyte–cumulus communication) using SAS, 14.3 software package (2017).

RESULTS AND DISCUSSIONS

In general, the result of the study (Table 2 and Figure 1) showed highly significant differences among the groups of matured oocytes ($p<0.001$) across the various parameters. An increase was observed in the rate of oocytes whose zona pellucida thickness exceeded 13 µm (63.39%). On the other hand, an increase in the rate of oocytes whose thickness of cumulus oophorus < 10 µm was observed compared to other groups (33,59%). The studied groups of oocytes were relatively similar in the rates of oocyte diameter. The oocytes whose diameter ranged between 100 and 120 µm achieved a slight increase (30.58%; the highest value) compared to the oocytes whose diameter was < 100 µm (29.54%). Of the total of 765 cultured oocytes, 305 oocytes (39.86%) were classified as "mature" (Figure2), while the rest 460 oocytes (60.14%) were classified as "degenerated" (Figure3).

Table. 2. Results obtained upon morphocytometric assessment after 27 hours of maturation

Parameter	Thickness(µm)	Oocytes after cultivation		Prop
		No.	%	
Zona pellucida	< 10	163	21,30%[a]	***
	10-12	117	15,29%[a]	
	>13	485	63,39%[b]	
Cumulus oophorus	< 10	257	33,59%[a]	***
	10-40	203	26,53%[a]	
	40-60	108	14,11%[b]	
	>60	197	25,75%[a]	
Oocytes diameter	< 100	226	29,54%[a]	***
	100-120	234	30,58%[a]	
	120-130	93	12,15%[b]	
	>130	212	27,71%[a]	

***p<0.001. Each subscript letter denotes a subset of case categories whose column proportions do not differ significantly from each other at the level p<0.05.

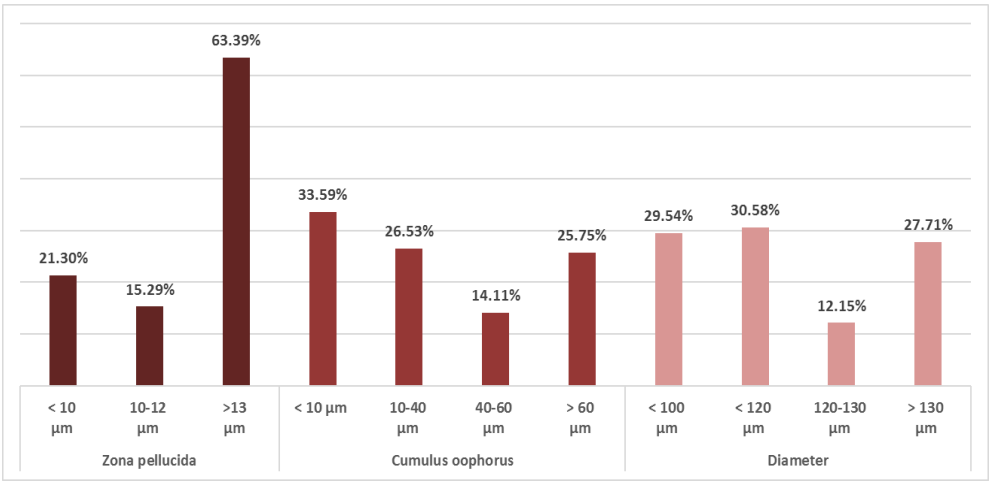

Figure1. Results of morphocytometric evaluation of equine oocytes across the various groups.

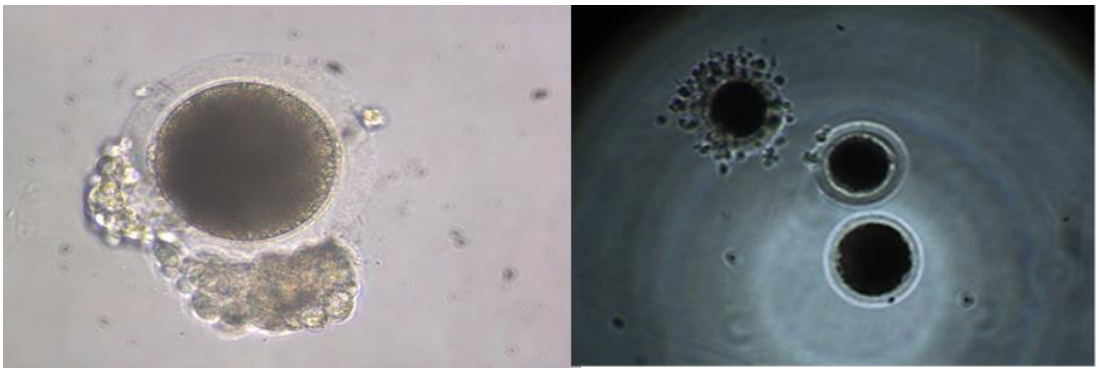

Figure 2. Mature equine oocytes based on morphological characteristics

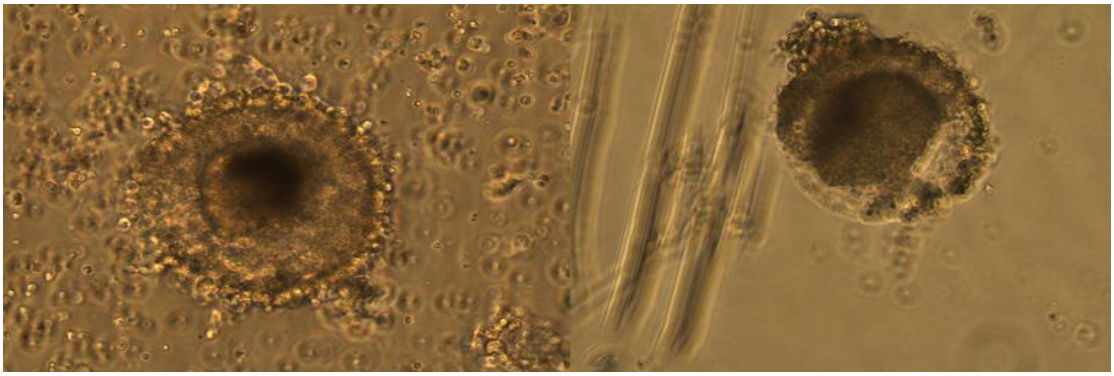

Figure 3. Degenerated equine oocytes according to morphological characteristics

To note, morphocytometric measurements have focused on establishing structural dimensions of oocytes after culture, especially oocyte diameter, cumulus and zona pellucida thickness and appearance of the first polar body.

The accurate measurements and the high fitness of the oocyte structures in morphometric analysis allowed us to complete the morphological examination that had been performed before, which led to the reclassification of the cultured oocytes into four quality classes (Figure 4 - a, b) as follows:

- Excellent mature oocytes: were within a diameter > 120 µm, intact pellucida ranging from 13 to14 µm, expanded cumulus to more than 50 µm of thickness, the first polar body with a length up to 30 µm and a width ranging from 20 to 24 µm, homogeneous cytoplasm and unified perivitelline space.
- Good mature oocytes: were within a diameter ranging from 110 to120 µm, membrane pellucida without destructions or rupture has the thicknessranging from 13 to 14 µm, compact cumulus ranging from 40 to50 µm, first polar body with a length ranging from 27 to 30 µm and width ranging from 20 to 24 µm, slightly granular cytoplasm and unified perivitelline space.

- Immature oocytes: were classified with diameter less than or equal to 100 µm, compact cumulus maximum 20 µm, zone pellucida without rupture has a thickness ranging from 11 to 12 µm, without first polar body and unified perivitelline space.
- Degenerate oocytes: were within a diameter < 100 µm, cumulus partial or complete denudation, broken zona pellucida or thickness < 10 µm, heterogeneous cytoplasm, grainy and ununified perivitelline space.

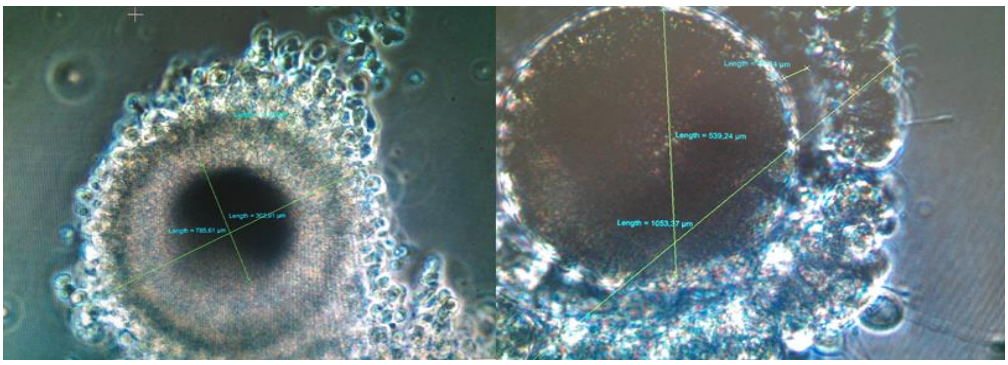

Figure.4. Morphocytometric evaluation of equine oocytes subjected to study

Of the total number of 765 cultured oocytes, 197 oocytes (25.75%) were classified as excellent mature, 108 oocytes (14.11%) as mature good and 203 oocytes (26.53%) as immature and 257 oocytes (33.59%) as degenerated.

The mechanism of oocyte maturation includes morphological and biochemical modifications. In the equine, most studies are histological and focus on nuclear maturation. The steps of the nuclear maturation of the equine oocyte (*in vivo* and *in vitro* conditions) are the breakdown of the nuclear membrane, the formation of the metaphase plate of the first meiotic division, the extrusion of the first polar body and the subsequent formation of the metaphase plate of the second meiotic division. During the maturation period (*in vivo* and *in vitro*) of equine oocytes, the cells from the cumulus oophorus expand. In *in vivo* case, after induction of ovulation, 83% and 100% of the oocytes have an expanded cumulus following 12 h and 35 h post maturation, respectively, (Claes et al.,2016). After *in vitro* culture, most oocytes have an expanded cumulus depending on the culture medium. In literature, although there have been numerous reports of the nuclear changes that occur during *in vitro* maturation of equine oocytes (Hinrichs et al., 2010a), little is known about the changes in the cytoplasm during maturation. Studies on this topic have been limited to preliminary observations of the migration of specific organelles (e.g., cortical granules) and the morphology of the meiotic spindle (Choi et al., 2003). However, it is clear that, as in other species, a series of complex cytoplasmic changes must accompany nuclear maturation if a developmentally competent oocyte is to result and that these events are largely dependent on a complete reorganisation of the oocyte cytoskeleton. Meiotic competence increases with increasing follicle size (Lewis et al., 2016), so the selection of larger follicles (e.g., selection of those follicles visible at the surface of the ovary) is related to an apparent increase in maturation rate. Oocytes from atretic follicles have greater meiotic competence than do those from viable follicles (Martino et al., 2014). Follicle atresia is associated with the expansion of the cumulus. The increase in meiotic competence with follicle size applies to both oocytes with compact (Cp) and expanded (Ex) cumuli. However, at each follicle size under 20 mm, the maturation rate for expanded oocytes is higher than that of compact oocytes (Sirard et al., 2001). Follicles in early atresia contain oocytes with the highest meiotic competence, and these oocytes may have either Cp or Ex cumuli(Hinrichset al., 2000). This probably accounts for a large portion of the confusion among laboratories in oocyte cumulus classification and its relationship to meiotic competence. Approximately 20% of collected oocytes are from follicles in early atresia, and because over 85% of oocytes in this category mature, the group into which they are placed (i.e., Cp or Ex) will appear to have a higher maturation rate. Cumulus appearance differs across oocytes aspirated from the follicle and oocytes scraped from the follicle wall, as aspiration tends to strip the majority of the cumulus from the oocyte (Viana et al., 2005) when only the corona radiata is present. Furthermore, it is not possible to determine whether the oocyte originally had a compact or an expanded cumulus. In addition, aspiration may preferentially collect oocytes from less viable follicles, in which the cumulus is not strongly attached to the follicle wall (Dell'Aquila et al., 2001). Even when recovering oocytes using a standardised technique such as follicle scraping, the criteria used for placement of oocytes in different categories, and in turn, the category of oocytes selected for culture vary among laboratories. In the laboratory, oocytes are collected from all visible follicles (as small as 2–3 mm of diameter) after serial slicing of ovaries. Only those oocytes that have both a compact granulosa layer and a compact cumulus are considered to be compact. In recent studies, the use of these

stringent criteria for cumulus classification resulted in 28–38% of oocytes being classified as compact, from 57 to 65% as expanded and from 4 to 8% as degenerating. The maturation rate of compact oocytes selected under such conditions ranges from 14 to 25% and 66 to 78% for expanded oocytes (Hinrichs and Schmidt, 2000). In contrast, other studies have reported up to 85% of collected oocytes as having compact cumuli (Choi et al., 2003; Claes et al.,2016), this percentage is commonly reported as over 50%. Many of the above factors are interrelated, e.g., the difference in the proportion of compact oocytes and their maturation rate observed among studies may be due not only to classification criteria but also to the follicles selected for the collection, as both the proportions of oocytes which are compact and the maturation rate of compact oocytes increase with follicle size (Scott et al., 2001; Iwata et al., 2004). Several studies have also found recently that immediate removal of oocytes from the ovary (within 1 h of ovary collection) is associated with higher maturation rates *in vitro* (Suzuki et al., 2000). Thus, a laboratory close to a slaughterhouse uses the aspiration method only for follicles visible on the surface of the ovary (reducing the time required to collect oocytes with a preference for collection from atretic follicles; i.e. larger follicles) which have non-strict criteria for classifying as compact, will report a much higher rate of oocyte maturation compared to a laboratory located some distance from an abattoir that collects oocytes from all follicles on the ovary surface (or only those under a certain size) by scraping the follicles and uses strict criteria to classify the oocytes as compact (Payton et al., 2000; Li et al., 2001).

CONCLUSIONS

It has been concluded from this study that slaughterhouses can be a good and cheap source of equine oocytes. Based on *in vitro* maturation of oocytes degrees (excellent, good, immature, degenerated), the oocytes rates differed according to the three studied parameters (cumulus oophorus, oocytes diameter and zona pellucida), so we recommend extensive studies on the possibility of the oocytes in *in vitro* fertilization according to previously studied parameters.

Author Contributions: Hussam Aryan collected the data and wrote the paper; Omar Mardenli performed the data analysis and general evaluation; Ioan Groza contributed analysis tools.

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Conflicts of Interest

The authors declare that they do not have any conflict of interest

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