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Effectiveness of the Ovarian Scraping Method in *In Vitro* Maturation of Equine Oocytes During the Breeding and Non-Breeding Seasons

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RESEARCH ARTICLE

Abstract

The current study aimed to study the feasibility of *in vitro* maturation of equine oocytes during the breeding and non-breeding seasons by using the ovarian scraping method. The collection of 752 ovaries resulted in 608 oocytes descending from 2824 ovarian follicles by the method of ovarian scraping at a rate of 21.53% and an average of 0.80 oocyte/ovary. During the breeding season, 432 oocytes were collected whereas 176 oocytes were collected in the non-breeding season. The results showed that the number of cultivable oocytes during the breeding season was 256 oocytes (59.25%), while the number of cultivable oocytes in the non-breeding one was 120 oocytes (68.18%) at the level of $p < 0.001$. The application of oocyte collecting by scraping method allowed obtaining a number of 608 oocytes, of which a number of 376 oocytes (61.84%) were cultivable, the rest of 232 oocytes (38.16%) were classified as uncultivable. It is concluded from the current study that the application of the ovarian scraping method in horses contributes to obtaining a high and encouraging rate of oocytes that can be used in *in vitro* embryo production programs.

Keywords: Equine; follicle; in vitro maturation; oocytes, ovarian scraping

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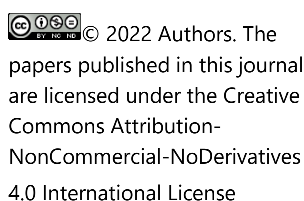

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INTRODUCTION

In equine, cloning by somatic nuclear transfer refers that oocytes derived from slaughterhouses can be used as receptor cells. The high number of gametes needed to create embryos and subsequently result in successful pregnancies explains why there is such a supply of oocytes. In the process of transferring cloned equine embryos, the study of Hall et al. (2013) showed that only 17 embryos were implantable out of 841 oocytes. However, only one birth was obtained. Thus, *in vitro* maturation rates can be affected by collection rates and oocyte quality, which positively affects pregnancy rates (Gonzalez et al., 2015). Even though the origin of the female donors and the quality of the oocytes are unclear, abattoir-slaughtered animals are a plentiful and fascinating supply of gametes. Because of this, these animals are regarded as top-notch experimental models and sources of information for teaching and using various biotechnologies (Oliveira et al., 2012). Comparing the equine species to other animals, the oocyte recovery rate is poor (Curcio et al., 2006). According to Dell'aquila et al., (2001), cumulus oophorus complexes (COCs) are more firmly adherent to the follicular wall in horses that has a direct impact on both *in vivo* and *in vitro* recovery rates.

Due to this, it is crucial to scrape or curettage the follicular wall while aspirating the follicles. These movements are intended to break the tight bonds that exist between the granulosa membrane and the follicular wall as well as between the cumulus cells and these membranes (Dell'aquila et al., 2003). Oocyte recovery methods include ovarian slicing, follicular wall curettage/scraping, ultrasound-guided follicular aspiration or Ovum Pick up (OPU). These methods can also be combined. Physical elements including vacuum pressure, the size of the needle and how the follicular wall scraping is done during follicular aspiration using the scraping technique are crucial for the procedure's success (Alm et al., 1997). Importantly, to achieve satisfactory results, technicians must be trained. Based on the features of the COCs, the potential for oocyte maturation, fertilization, and embryo development rates is calculated. Under a stereomicroscope, these cells are evaluated subjectively, and the presence and/or extension of the cumulus are graded according to the severity. The distinctions between the two species are sometimes overlooked in this strategy because it also has a comparative element, particularly when it comes to the bovine species (Lagutina et al., 2005). The main purpose of this study was to evaluate and determine the effect of the method of equine oocytes collection using the method of ovarian scraping on the yield of *in vitro*-produced embryos. Accordingly, subsidiary, the morphological evaluation of equine oocytes and their classification according to structural aspects has been proposed.

MATERIALS AND METHODS

Animals, ovaries collection, transportation and experimental design

The study was carried out on 752ovaries collected from 376 mares aged between 4 and 19 years old. The mares were slaughtered in the slaughterhouses in Vințu de jos (Alba Iulia) and Cetina (Baia Mare), Romania. The ovaries were collected 10 minutes post slaughtering of the mares and transported to the laboratory in 0.9% sodium chloride solution supplemented with 100 µg/ml streptomycin and 100 IU/ml penicillin or in phosphate buffer saline (PBS) solution supplemented with antibiotics at a temperature of 30 - 33 ^{0}C. A maximum interval of 3-4 hours between the ovarian collection and the collection of the oocytes was imposed. The ovaries were washed in sterile physiological serum (Figure 1).

Figure 1. Collected equine ovaries included in the study

Based on a previous paper (Aryan et al., 2015), the size of the collected ovaries, including their follicles, was measured using a ruler inserted in millimeters and classified according to size into three classes: Class I (> 30 mm), Class II (from 20 to 30 mm) and Class III (<20 mm). From the total of 752 ovaries used in the study, 424 ovaries were collected in the breeding season and 328 in the non-breeding season.

Oocytes collection

The ovarian scraping technique was applied to the surfaces of the follicles to obtain the oocytes. By resorting to the sterile scalpel blades, the follicles with a diameter greater than 10 mm were sectioned. Next, the superficial scraping was performed inside the follicle to ensure the release of the COCs with the follicular fluid simultaneously (Figure 2).

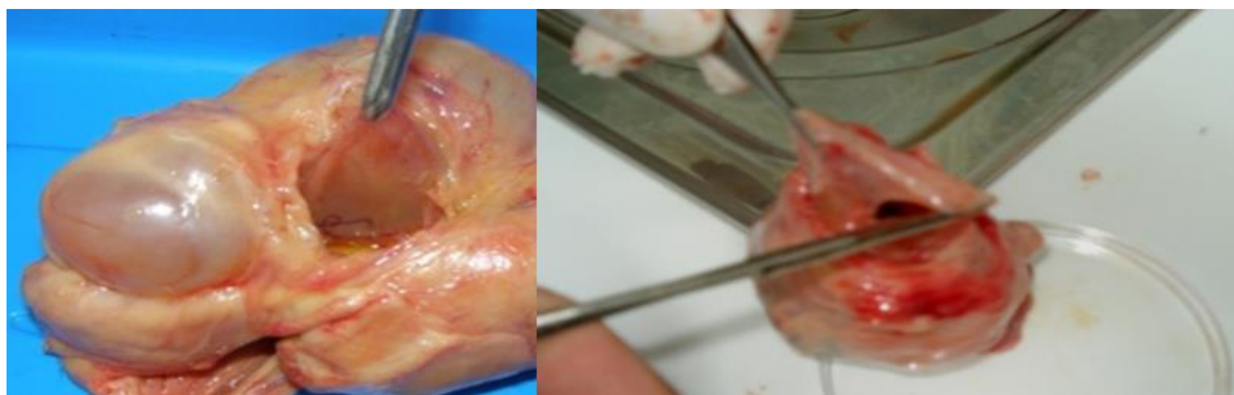

Figure 2. Collecting of equine oocytes by scraping method

Following collection at 22 ^{0}C, the liquid was examined under a stereoscope to identify and select the oocytes. To favor oocyte sedimentation, the collected follicular fluid was kept in tubes for 5 minutes at laboratory temperature. After removing the supernatant, the sediment was examined under a stereoscope to identify the oocytes as well as to establish the morphological characters. The sampling and selection of oocytes across the follicular fluid were carried out by resorting to the sterile glass pipettes of a diameter of 50 μm (Einmal-Mikropipetten pipettes), which allowed to avoid mechanical destruction at the cellular level.

Morphological assessment

The morphological evaluation of the oocytes was also carried out with the Nikon inverted phase microscope equipped with a video camera in the Department of Reproduction, Obstetrics and Veterinary Gynecology to classify and select the oocytes for in vitro maturation. The morphological characteristics that were the basis for assessing the integrity and viability of equine oocytes were established according to appearance of the zona pellucida (sphericity and integrity), appearance of cumulus oophorus (number of cumulus cells and compaction), appearance of the cytoplasm (texture of the cytoplasm, presence of vacuoles, granulation, opacity and homogeneity) and appearance of the perivitelline space (presence of detached cells, thickness and uniformity).

Statistical analysis

Pearson's chi-square test was conducted on the data of the experiment to demonstrate the significant differences among the groups applied. Fisher's exact test was used for further rates comparison. All the analysis was performed using SAS, 14.3 software package (2017).

RESULTS

A significant difference was observed ($p<0.001$) in collection rates during the breeding and non-breeding seasons (Table1). Figure 3 shows that collection rate during the breeding season was 25.5% with an average of 1.01 oocytes /ovary (Group I) whereas the collection rate during the non- breeding season was 15.4% with an average of 0.53 oocytes/ovary (Group II). The examination of the collected follicular fluid allowed identifying and selecting of oocytes according to the structural elements, thus being able to establish their classification in the two categories of quality:

Table1. Rates of cultivable and non-cultivable equine oocytes during the breeding and non-breeding seasons

Group	Collected oocytes (No.)	Cultivable oocytes		Non-cultivable oocytes	
		No.	%	No.	%
Season	432	256	59,25[a]	176	40,75[a]
Non-breeding season	176	120	68,18[b]	56	31,82[b]
Total number	608	376	61,84	232	38,16
p		<0.001		<0.001	

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

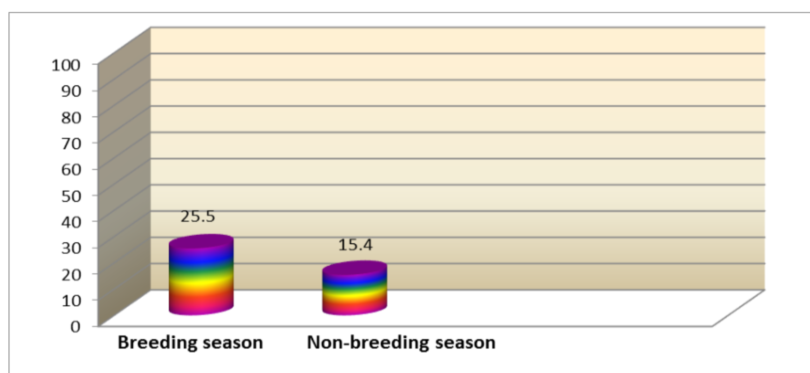

Figure 3. Collection rate of oocytes obtained by scraping method in breeding and non-breeding season

A total number of 376 oocytes were ready for cultivation as they presented a compact cumulus with many layers of cells, fine and densely granulated cytoplasm, an intact zona pellucida and a unified perivitelline space. The averages of non-cultivable oocytes reached the values 40.75% (Group I) and 31.82% (Group II); $p<0.05$. Applying the scraping method during the breeding and non-breeding seasons allowed obtaining a number of 608 oocytes, of which a number of 376 oocytes (61.84%) were cultivable, the rest 232 oocytes (38.16%) were classified as and non-cultivable (Figure 4). In general, the collection rate was 21.53% and average of 0.80 oocytes/ovary. A total number of 432 and 176 oocytes were collected in the breeding and non-breeding seasons. Of the 432 oocytes collected in the breeding season, 256 oocytes (59.25%) were cultivable, and 176 oocytes (40.75%) were non-cultivable. Of the 176 oocytes collected in the non-breeding season, 120 oocytes (68.18%) were cultivable, and 56 oocytes (31.82%) were non-cultivable. In addition, a number of 376 oocytes (61.84%) were suitable for cultivation, the rest of 232 oocytes (38.16%) were classified as non-cultivable.

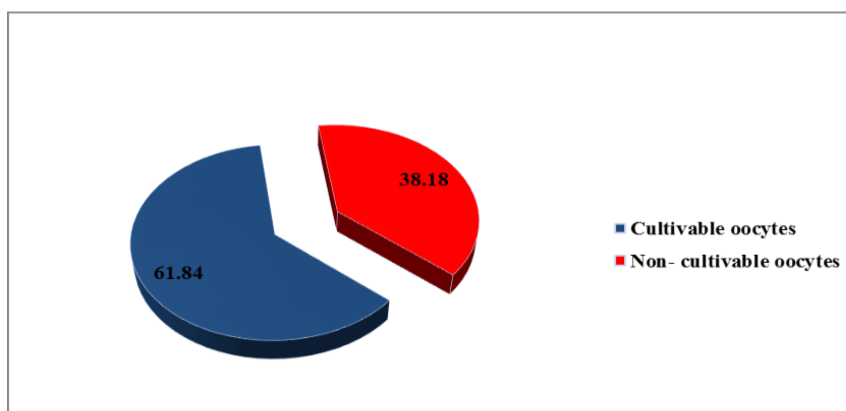

Figure 4. Cultivable and non- cultivable oocytes collected by ovarian scraping method

Depending on the morphological characters identified by using the stereoloupe and the inverted microscope (Nikon Eclipse TS100, inverted phase contrast, Nikon objectives), the cultivable oocytes presented a compact cumulus and that contained two or more layers of cumulus cells, homogeneous cytoplasm, slightly granulated, intact pellucida without destruction or ruptures and unified perivitelline space (Figure 5). The non-cultivable oocytes showed partial or total denudation, heterogeneous, deformed and strongly granulated cytoplasm, broken zona pellucida and uneven perivitelline space (Figure 6).

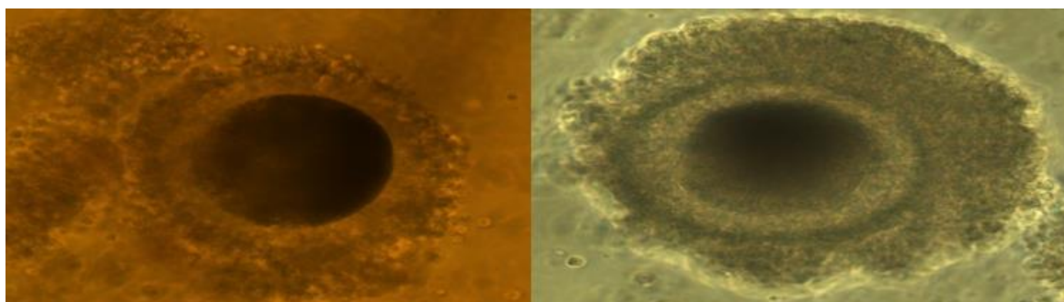

Figure 5. Cultivable equine oocytes at 20x magnifications

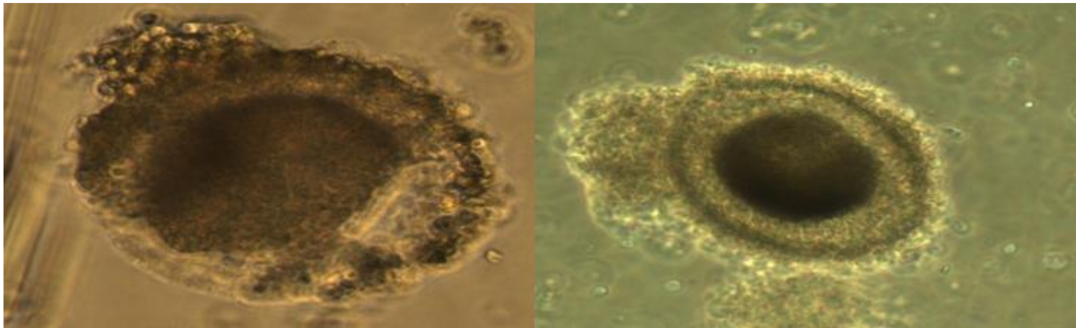

Figure 6. Non-cultivable equine oocytes at 20x magnifications

DISCUSSION

Perhaps the most important factors that mainly control the effectiveness and success of the *in vitro* embryo production technique are the process of collecting oocytes and the reproductive status of the donor females before ovaries are collected. Logically, the results obtained in this study (Figures 1, 2, 3, 4, 5 and 6) can be traced back to a set of explanations and findings in many related studies. Taking into account the species, reproductive activity and the time of year of the collection process (collecting the ovaries), oocytes capable of completing nuclear and cytoplasmic maturation are governed by a highly complex scenario of hormonal activity. Normally, this activity is manifested by a series of changes in the pathways and timing of action of the hormone related to the maturation process. Not only that, in the dialectic of the dynamic follicular wave, the group of follicles in the ovary will be subjected to a series of hormonal activities (LH surge) that will cause the emergence of the dominant follicle that includes an oocyte qualified to complete the processes of maturatin and subsequent nuclear division in preparation for a successful fertilization (Ginther et al., 1996; Aryan et al., 2016; Mardenli et al., 2020; Mardenli et al., 2021) The previous scenario described is the reason for the emergence of different degrees of developmental competence of the oocytes, which in turn determines whether the oocyte is cultivable or not (Mihm et al., 2000). In literature, there were many methods of oocyte collection (e.g., slicing, aspiration and ovarian scraping), which led to obtaining highly dispersed rates due to the type of oocytes targeted (mature or immature) according to each method. In general, the method of collecting by ovarian scraping is characterized as the most important method in collecting the largest possible number of oocytes. By choosing the technique used for collecting oocytes, the following considerations were taken into account:

- Improving the methods of identifying the follicles across the surface and inside the ovarian cortex.
- The full recovery of the follicular fluid and the increase of oocyte recovery rate.
- The intervention must be atraumatic regarding the oocyte existing inside the follicles.
- The cost price should be affordable.

In this study, the follicular wall scraping technique was used to aspirate follicles from 752 ovaries, yielding an average of 0.80 retrieved oocytes per ovary (2824 follicles/ 608 oocytes). Accordingly, Choi et al. (2001) used the same approach and obtained an average of 1.8 oocytes/ovary. Given that similar numbers of ovaries (311 to 517) were reported in other investigations, the sample size employed in this study to assess the follicular wall scraping technique was deemed adequate (Berteroet al., 2017; Cremonesi et al., 2010). Oocyte recovery rates of 63 % and 56 %, respectively, were observed by Carnevale et al. (2006) when comparing the rates achieved via follicular wall scraping and curettage. The success rate (61, 84 %) and a number of 376 oocytes were considered as ready for *in vitro* maturation; show the potential for this technique to be used more successfully. With the application of the follicular wall curettage approach, high oocyte recovery rates and the recovery of intact COCs have been observed, permitting the classification of oocytes quickly after their recovery (Hinrichs et al., 2005b; Fair et al., 2003). This was further supported by the current study's use of the follicular wall scraping technique. According to the same study, the collected oocytes in the non-breeding season were fewer but higher quality und higher rate of cultivable oocytes. From 176 oocytes, a number of 120 oocytes were cultivable (68, 18%). The follicular wall curettage approach requires the isolation of each follicle, making it a time-consuming and difficult procedure (Galli et al., 2007). Cremonesi et al. (2010) reported that it typically took 4 to 5 hours to use a vacuum pump to scrape the follicular wall. Therefore, to increase the technique's effectiveness and retrieve oocytes of higher quality, scraping of the follicular wall is required in both *in vivo* and *in vitro* procedures (Krisher et al., 2004). To recover more COCs, the team must have received prior training in addition to selecting the best technique. Given that cloning via nuclear transfer necessitates a large number of oocytes, the follicular wall scraping approach employing needles and syringes is an intriguing way to collect oocytes (Valenzuela et al., 2017). This procedure can be carried out quickly and is linked to the recovery of intact CCOs, offering encouraging maturation, blastocyst and pregnancy rates. Our results are in agreement with most studies in which the oocytes collection rates ranged from 5 to 95% and the rates

of cultivable oocytes (from 5 to 90%) according to reproductive activity and estrus. The study conducted by Karami et al. 2011 showed that the rates of cultivable ovine oocytes collected by aspiration method ranged between 67% and 92% across five types of maturation mediums (human menopausal serum, estrus sheep serum, estrus goat serum, ovine follicular fluid and bovine follicular fluid). In another study, the rates of cultivable ovine oocytes collected by three methods (aspiration, slicing and puncture) ranged between 22% and 59% (Majeed et al. 2022). The rates of cultivable goat oocytes collected by puncture, slicing and aspiration methods reached 56%, 60% and 74%, respectively (Hoque et al. 2011). In addition to the foregoing, reference is made to a group of factors that are associated with the collection methods, such as female age, nutritional status, hormonal treatment methods and types of superovulation. The mentioned factors can give a specific explanation for the proportions of oocytes collected according to each method. Therefore, the collection method may play a role that is not a small matter in determining the detailed determination of each of the aforementioned factors (Storey, 1995; Stubbings et al., 1988; Swain et al., 2001; Tatemoto et al., 2001; Summers and Biggers, 2003).

CONCLUSION

The results of the current study showed that the use of the ovarian scraping method to collect equine oocytes during the breeding and non-breeding seasons led to obtaining high rates of oocytes that can be entered in the *in vitro* embryo production programs.

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Conflicts of Interest

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

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