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Technical Note

Collection and *in vitro* maturation of the equine oocyte: The first attempt in Iran

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Abstract Nowadays, assisted reproduction has become an essential part of the management of horse reproduction in different parts of the world. *In vitro* production of equine embryos requires established techniques including oocyte collection, *in vitro* oocyte maturation and intracytoplasmic sperm injection. No report is available on *in vitro* oocyte maturation of horse oocytes in Iran. Ovaries were immediately collected from newly dead mares of different ages, and transported to the laboratory. The visible follicles were opened using a scalpel blade and the granulosa layers of the follicle wall were scraped from the follicle. Then, the ovaries were cut in 5 mm sections to collect more oocytes from follicles within the ovarian stroma. The oocytes were cultured in TCM-199 medium, supplemented with 10% fetal calf serum and hormones, in a CO_2 incubator at 38.5 ^{0}C for 30 h. In addition, using a home assembly devised ovum pick-up (OPU) system, five attempts were made to collect oocytes from preovulatory follicles in four mares. After 30 h in culture, the denuded oocytes were fixed and stained with aceto-orcein to determine the nuclear maturation of the oocytes. Out of 29 cultured oocytes from dead mares, 15 (51.7%) oocytes reached the metaphase II. Further, of five OPU operations on live mares, one compact COC suitable for culture and one degenerate COC were collected. The results of this report describe the feasibility of ovum pick-up and oocyte collection from either live mares or immediately after death of the animal and further successful *in vitro* maturation of the equine oocytes.

Keywords: oocytes, horse, *in vitro* maturation, follicles, ovum pick-up

Paper type: Technical Note

Introduction

In horses, albeit later but like in other farm animal species, advanced assisted reproductive techniques have now gained acceptance in many equine industries particularly in the last decade (Stout, 2020). These techniques are mainly based on the collection of immature oocytes which are transferred to another location for intracytoplasmic sperm injection (ICSI). *In vitro* fertilization in horses, unlike other farm animal species, requires the delicate technique of ICSI. The ICSI-produced embryos may then be transferred either on the site or transported

to another region, or cryopreserved. The ICSI may also be used for subfertile stallions or mares. In Iran, the horse industry has remarkably progressed particularly in the last two decades; however, application of the assisted reproductive techniques has been mainly limited to the artificial insemination of either fresh, diluted or frozen semen. There are scarce researches on the advanced assisted reproductive techniques including embryo transfer (Bolourchi et al., 1997), ovum pick-up (Ahmadi and Javidpour, 1997) and transfer into the oviduct (Nazem and Abrisham-Chian, 2018) in Iran.

To perform a successful ICSI, the immature oocytes need to be cultured *in vitro* to reach the metaphase II stage of meiotic division which is called *in vitro* maturation (IVM). *In vitro* maturation of the horse oocyte has resulted in production of metaphase II stage from 32 (Carnevale, 2016) to 67 (Lewis et al., 2016) percent in different laboratories. One important obstacle for routine collection of horse oocytes is the lack of horse abattoir in many countries, including Iran. For this reason, the techniques for ovum pick-up have been successfully developed using transvaginal ultrasonography. Ovum pick-up, albeit an expensive technique, is becoming the method of choice to collect horse oocytes in many well-equipped clinics in the world.

A major limitation for research on *in vitro* oocyte maturation and fertilization in the horse is the difficulties in collecting large numbers of oocytes. Various methods including aspiration, scraping the follicular wall, slicing the ovaries or a combination of these have been used to collect oocytes from mare ovaries in the laboratory (Alm et al., 1997; Colleoni et al., 2007). Of these methods, the combined method of scraping the follicular wall and slicing the ovaries is the most common to obtain more oocytes with better quality per ovary (Morris et al., 2018). No information is available regarding the *in vitro* maturation of the horse oocyte in Iran. The present report describes the results of our first attempt to collect and mature *in vitro* the oocyte from live or newly dead mares.

Materials and methods

Oocyte collections from newly dead mares

The ovaries form four adult mares and two fillies were collected immediately after death. The animals had died due to problems such as colic and dystocia or euthanized due to leg fractures. Each ovary was placed into an individual plastic bag and transported in a container in isotonic saline (30 to 35 ^{0}C) to the laboratory within 2 to 4 h. Upon reaching the laboratory, ovaries were cleaned by trimming the adnexa with scissors and washed with warm sterile saline. External follicles were opened with a scalpel and the follicular wall was scraped using a 16-gauge needle fitted with a 20 mL syringe. Then, the ovaries were cut in 5 mm sections to collect more oocytes from the internal follicles. The follicular contents were transferred to a 100 mL petri dish, washed into petri dishes containing TCM-199 supplemented with 10 units/mL heparin (Caspian, Iran), and examined under a stereomicroscope (10 ×). Cumulus-oocyte complexes (COCs) were classified and held in the TCM-199 with 10 units/mL heparin. Only the COCs with either compact cumulus (oocytes surrounded by compact cumulus investment) or oocytes with the expanded cumulus (oocytes with completely or partially expanded cumulus cells (Fig. 1)) were used for *in vitro* culture (Mlodawska and Okolski, 2009).

Transvaginal ultrasound-guided preovulatory follicle aspiration

A real-time ultrasound scanner (SIUI 900, China) equipped with a 5 MHz convex array human transducer matched with a 12-G double lumen steel needle (50 cm in length, 1 mm internal diameter, Minitube, Germany) covered with a hard polyethylene tube was used. This system was home assembly devised and adapted at the Department of Animal Reproduction, Shiraz Veterinary School, to aspirate the preovulatory follicles in mares. A vacuum pump was also attached to the needle, and the aspiration negative pressure was set at 150 mmHg. Four mares (5 to 15 years of age) in estrus were used for the transvaginal oocyte collection. Prior to the day of aspiration, the mares were examined by transrectal palpation and ultrasound to evaluate the uterine edema and also determine the size and location of the preovulatory follicle. The mares were administered 2500 IU hCG (Pregnyl, Organon, The Netherlands) intramuscularly to induce ovulation of the preovulatory follicles greater than 35 mm in size 24 h before oocyte collection. Prior to the follicle aspiration, mares were restrained in stocks and sedated with 0.6 mg/kg Xylazine (iv, Interchemie, The Netherlands), as well as 0.9 mg/kg N-butylscopalammonium bromide (iv, Exir, Iran) for rectal relaxation. The ultrasound transducer was inserted into the vagina, and the ovary was attracted transrectally to lie against the vaginal wall near the transducer. The follicles were aspirated by inserting the double lumen needle into the follicular cavity while viewing through the mon-

itor of the ultrasound scanner, then massaged per rectum and flushed continuously with 150 to 200 mL of ringer solution (37 ^{0}C) containing 10 units/mL heparin and 2% fetal calf serum (Ortis and Foss, 2013). The follicle flushing was repeated 3 to 5 times in each mare. After the follicle aspiration, the fluid was immediately transported to the laboratory and examined under a stereomicroscope for recovery of the COCs.

In vitro culture of oocytes and evaluation of nuclear maturation

All chemicals used for *in vitro* culture of oocytes were purchased from Sigma-Aldrich, USA. Only compact and expanded oocytes with excellent qualities were cultured for *in vitro* maturation. Oocytes were cultured in 500 µL of TCM-199 supplemented with 10% fetal calf serum and 5 µg/mL FSH and 25 µg/mL gentamicin for 30 h in humidified atmosphere of 5% CO_2 in air at 38.5 ^{0}C. The time between the death of the animals and onset of *in vitro* culture ranged from 2 to 4 h. After culture, oocytes were denuded of cumulus cells using a micropipette, fixed in acid alcohol for approximately 24 h and then stained with aceto-orcein. Nuclear maturation was determined under a contrast phase microscope (Hund, Wetzlar, Germany) at 400 magnifications (Morris, 2018).

Statistical analysis

The Chi-squared test was used to compare the percentages of good quality oocytes for culture that recovered from the external and internal ovarian follicles. P values less than 0.05 were considered statistically significant.

Results

On the whole, 54 COCs in different qualities were recovered from the 8 ovaries of the adult mares. On average, each ovary yielded 6.7 COCs with a mean number of 5.7 oocytes suitable for culture. Out of 10 oocytes from the external follicles of the ovaries, 8 good quality COCs were recovered while using slicing method on internal follicles 46 oocytes were obtained of which 38 were suitable for culture (80.0 vs 82.5%; P>0.05). Of 29 cultured COCs, 15 (51.7%) reached the MII stage of meiotic resumption (Fig. 2) after 30 h culture. The ovaries of fillies were static and small in size with only four small-sized follicles (<15mm) confirming the prepubertal anestrous stage in these animals. Two good quality and one degenerated COCs were recovered from the four external follicles of young mares. Good quality COCs of the fillies

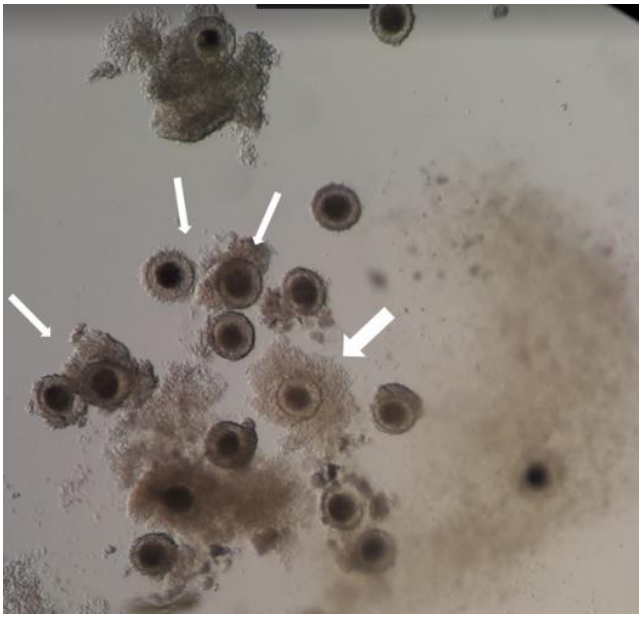

Figure 1. Representative pictures of various equine oocytes collected using a combined method of scraping the follicular wall and slicing the ovaries. Flat arrow shows an oocyte with expanded cumulus cells and narrow arrows show compact oocytes suitable for culture, × 50.

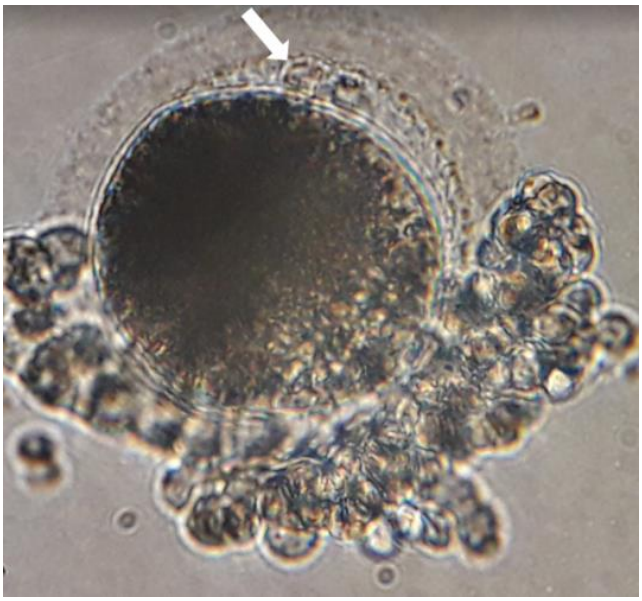

Figure 2. A matured equine oocyte with the extruded first polar body (arrow) in the peri-vitelline space in metaphase II, × 100.

showed signs of complete expansion of the cumulus cells after 30 h culture.

Of five OPU operations on live mares (one mare was used two times), two COCs (one good quality compact, Fig. 3) and one degenerate COCs) were collected. The COCs were collected from the preovulatory follicles

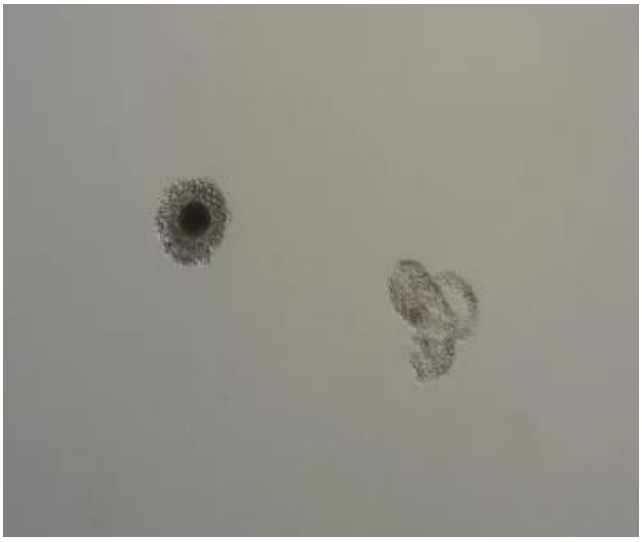

Figure 3. An immature compact oocyte collected from a 38 mm preovulatory follicle using the ovum pick-up system, × 50.

≥ 38 mm.

Discussion

The results of the present study showed that using the combined method of scraping the follicular wall and slicing the ovaries resulted in obtaining high number of oocytes with culturable quality (5.7 oocytes per ovary). Our results are comparable with Choi et al. (1993) that obtained 4.14 oocytes per ovary when the ovaries were sliced and washed. Using methods such as aspiration or scraping the follicular wall alone did not result in the recovery of high number of oocytes (Alvarenga and Landim-Alvarenga, 2009). The low recovery rate using aspiration method is due to the fact that in mares, the oocyte is firmly attached to the follicular wall which interferes with the efficiency of oocyte recovery (Hinrichs, 2010). The number and quality of the oocytes that are collected depend on the season, the stage of the estrous cycle, nutrition, age and the collection method (Carnevale, 2016). The percentage of good quality oocytes retrieved from the external follicles was not different from that of the internal follicles (80 vs 82.5%). This was expected as the mares were in their reproductive season.

The metaphase II stage of nuclear maturation in the horse oocyte, and also in other farm animals, is characterized by the presence of metaphase chromosomes in the periphery of the ooplasm and presence of an extruded polar body in the peri-vitelline space (Alvarenga and Landim-Alvarenga, 2009). In the present study, by the end of the culture period, 51.7% of the cultured oocytes reached the metaphase II. Hinrich (2010) reported that *in vitro* nuclear maturation rates in the mare oocyte can be from 20 in compact to 65% or even higher in the expanded oocytes. The optimum *in vitro* culture time for the mare oocytes ranges from 24 to 30h for expanded oocytes to 30 to 36h for compact oocytes (Hinrichs et al., 2005; Morris, 2018). The reason that our nuclear maturation rate is somewhat in between the reported maturation rates could be the 30h culture time and the pooled oocytes with different morphologies. The presence of cumulus cells, addition of gonadotropins to the maturation media, oocyte morphology, the size and physiologic status of the follicle and time of culture have been considered as the main factors that may affect meiotic resumption of the oocytes (Alvarenga and Landim-Alvarenga, 2009; Mlodawska and Okolski, 2009; Carnevale, 2016). The results of the present study further showed an oocyte recovery rate of 40% out of five ovum pick-up operations in mares with preovulatory follicles ≥ 38 mm. By using a home assembly devised ovum pick-up system, an acceptable recovery rate of the oocyte was obtained from live mares in the present study. Other researchers, using a well-equipped ovum pick-up system, reported a recovery rate of 50% from immature to 85% from mature follicles (Ortis and Foss, 2013; Morris, 2018). More researches using improved ovum pick-up system are now required to introduce this highly demanding technique to the horse industry in Iran.

Conclusions

In this communication, we described the feasibility of ovum pick-up and oocyte collection from either live mares or immediately after death of the animal along with successful *in vitro* maturation of the oocytes.

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