Universitat Autònoma de Barcelona

EFFICIENCY OF OOCYTE FOLLICULAR ASPIRATION (OPU) TO OBTAIN EMBRYOS BY INTRACITOPLASMATIC SPERM INJECTION (ICSI) IN THE MARE AND COMPARISON WITH DONKEY RESULTS

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1. INTRODUCTION

In vitro embryo production via ovum pick up (OPU) and intracytoplasmatic sperm injection (ICSI) is a breeding technique that has become very popular during last years, mainly because *in vitro* fecundation (IVF) does not work in horses yet (Leemans et al., 2016) and therefore it is the newest way to maximize reproduction efficiency and genetic preservation (Squires, 2020). OPU-ICSI programmes are an effective means of obtaining foals from mares that do not provide embryos for embryo transfer and/or are unable to successfully complete any pregnancy due to chronic endometritis, cervical defects, age, damage to the reproductive tract or any other problem that induces poor fertility (Herrera, 2018; Morris, 2018). Moreover, ICSI is also indicated for producing embryos from stallions with poor semen quality or quantity that would not fertilize any oocyte by their selves due to age, genetic problems, etc. (Roels et al., 2018). Other use of this technique is to optimize frozen semen from death stallions, by dilution of standard straw of frozen semen that can be sectioned into multiple ICSI doses (Rader et al., 2016). It is also a reproductive technique to consider for competition mares as it can be done at any time of the year and does not need many days of training rest. However, it is more expensive than conventional embryo transfer (Salamone et al., 2017; Rader et al., 2016). Although OPU-ICSI is getting more and more popular in equine breeders, there is anything reported about it in donkeys. The few studies focused in OPU in donkeys report the chemical activation of oocytes followed by IVF and vitrification of early embryos (Abdoon et al., 2018; Deleuze et al., 2018). At this point it is relevant to mention that despite the great improve in donkey semen cryopreservation, the use of thawed semen for artificial insemination produces low results of pregnancy in jennies (Álvarez et al., 2019; Canisso et al., 2011; Miró et al., 2020; Oliveira et al., 2012; Vidament et al., 2009). The development of new reproductive techniques as *in vitro* production of embryos in donkeys could satisfy the necessity to conserve some donkey breeds, as many of them are in critical status, and to optimize the use of donkeys in other areas (milk production, rural tourism, cosmetics…) (Camillo et al., 2018).

The use of transvaginal ultrasound guided OPU to obtain oocytes for fertilization by ICSI has generated much enthusiasm in commercial equine breeding as is much less invasive than flank surgery, becoming into the method of choice today (Rodriguez et al., 2021). It has been demonstrated that 5-12 oocytes can be recovered per OPU session depending on the number of follicles presents and other mare factors (Galli et al., 2014).

After first demonstration of successful pregnancy from ICSI horse embryo (Galli et al., 2007) several results have been published to date, but many of them are controversial. In recent years, oocyte collection has been reported from 50-60% (Cuervo-Arango et al., 2019b; Rader et al., 2016; Claes et al., 2022; Barbacini et al., 2009), whereas oocyte maturation varies from 59-66% (Claes et al., 2022; Rader et al., 2016). The more successful rates of blastocyst per injected oocyte were 21.2% (Cuervo-Arango et al., 2019b) and 23.0% (Rader et al., 2016). However, the most common is from 10.0-17.6% (Meyers et al., 2019; Barbacini et al., 2009.), and in some individual cases it was up to 46.7% (Cuervo-Arango et al., 2019b). Furthermore, when talking about overall results per OPU session it has never been described more than 1.7 blastocysts/ OPU (Cuervo-Arango et al., 2019b). Results of OPU implemented in jennies yields in more variable recovery percentages from 34-76% (Deleuze et al., 2018).

The aim of this study was to evaluate the efficiency of OPU-ICSI procedures in horses performed at the "*Servei de reproduction equina de la Universitat Autònoma de Barcelona*" and to compare it with the results obtained with donkeys in the same place.

2. MATERIAL AND METHODS

2.1. POPULATION

A total of seven mares were used in this study. The population of mares studied included one with good genetic without any known fertility problem at that moment to others that had subfertility results in the past reproductive seasons. Different breeds of mares were also present in the study. In the case of donkeys, only five Catalonian jennies were used with no reproductive problem knew. In some animals, more than one OPU session was conducted.

2.2. OPU

The oocyte recovery was done by aspiration of the immature follicles of > 10 mm. The number of oocytes recovered depends upon the number of follicles present able to be aspirated. Oocytes were collected by transvaginal ultrasound guided follicular aspiration technique. The mares and jennies were placed in palpation stocks and sedated using Detomidine hydrochloride (0.01mg/kg IV for mares, 0.02 mg/kg for jennies) and Butorphanol tartrate (0.02mg/kg IV for jennies, 0.03 mg/kg for jennies). They were also medicated with Butilscopolamine (0.15 mg/kg IV for mares, 0.12 mg/kg for jennie), Flunixin Meglumine (1.1mg/kg) and with a prophylactic antibiotic protocol consisting in Gentamicin (6.6 mg/kg IV) some minutes before the process. The tail was tied up, the rectum was evacuated, and the urinary bladder was proved and emptied of urine. The perineum and vulva were scrubbed three times with neutral soap.

A total of 26 OPU sessions were done with an ultrasound transducer with a section probe (E3123, MyLabGamma, Esaote®, Genova, Italy) placed in a holder with a follicular aspiration double lumen needle of 12G x 25" attached to a double vacuum pump (Minitüb, Tiefenbach, Germany). All together was passed transvaginal, through the vaginal wall and into a follicle. The ovary was grabbed via rectum and manipulated to bring it to the vaginal wall and positioned correctly in order to facilitate the follicular punction. Penetration of the vaginal wall, ovary and follicular wall were done in each follicular punction facilitated by firm pressure of the ultrasound probe against the vaginal wall.

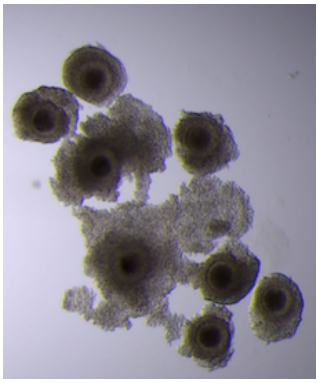

Figure 1: Donkey cumulus-oocytes obtained after OPU (image courtesy by Jordi Miró)

Once the needle was seen by echography image into the follicle, the follicle was flushed 5-10 times with a commercial recovery medium (Equiflush®, Minitüb, Tiefenbach, Germany) that contains Sterile water, Hepes, D-glucose, Na-pyruvate, Kanamycin, Gentamicin and supplemented with Heparin (500UI/ml Heparinasodica®, ROVI, Madrid, Spain) prewarmed at 37ºC (Cuervo-Arango et al., 2019). It is important to rotate vigorously the needle to dislodge the oocyte from the follicular wall during the flushing (Ortis & Foss, 2013). All the fluid obtained was maintained at 37ºC into the vacuum equipment (Cuervo-

Arango et al., 2019b). Once the aspiration of all oocytes was completed the collected fluid was transported to the laboratory for the oocyte searching process. The collected fluid was filtered through a sterile 70 mm embryo filter (EmCon®; IMV Technologies,L'Aigle, France), and the filtered content was evaluated under a stereomicroscope (SMZ800 N, Nikon Corporation, Tokyo, Japan) to identify the oocytes. The oocytes were placed in a 2 mL embryo holding media (Minitube, Bayern, Germany) and transported to the ICSI laboratory.

2.3. OOCYTE MATURATION

Oocyte *in vitro* maturation was performed for 36-38 hours in 100 μL drops of bicarbonate-buffered Tissue Culture Medium (TCM-199 1,150-059, Thermo Fisher Scientific,Waltham, USA) supplemented with 10% v/v fetal bovine serum (FBS; 0091148 8, Thermo Fisher Scientific, Waltham, USA)]and 1 μL/mL Insulin-Transferrin-Selenium (51300044, Thermo Fisher Scientific, Waltham, USA), 1 mM sodium pyruvate 100 mM cysteamine (M9768), 10μg/mL follicle stimulating hormone (Folltropin-V, Vetoquinol Especialidades Veterinarias, Madrid, Spain), and 25 μ g/mL gentamycin (15710064, Thermo Fisher Scientific, Waltham, USA) under mineral oil (ART-4008PA, Origio Oil for Tissue Culture, Cooper Surgical Fertility Companies, Målov, Denmark) in 5% CO2 in humidified air at 38.2ºC. For donkey oocytes a 10% of donkey preovulatory follicular fluid (PFF) was used instead of FBS in 24 oocytes.

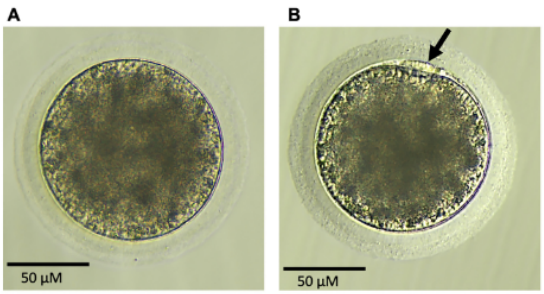

Figure 2: Immature oocyte (A) and mature oocyte (B) from jennies (sedated by Jordi Miró).

2.4 MANAGEMENT OF OOCYTES

After the maturation period, the dish containing the oocytes was removed from the incubator, and the oocytes were evaluated using a dissection microscope. The cumulus cells were mechanically removed in G-MOPSTM medium (10.130, Vitrolife, Goteborg, Sweden) to assess the presence of the first polar body, which means that they have successfully maturated into MII oocytes (Hinrichs, 2010; Rodríguez et al., 2019; Metcalf et al., 2020). The oocytes with intact cytoplasmatic membrane without any visible polar body were classified as immature. Only those oocytes where the first polar body was observed were placed back into maturation droplets and into the incubator to wait for ICSI.

2.5 ICSI

ICSI is a micromanipulation technique that consists of an injection of a single spermatozoon into the cytoplasm of a mature oocyte. It involves the use of an inverted microscope (Nikon Eclipse Ti2-A, Nikon Corporation, Tokyo, Japan) and a micromanipulation system (Transferman 4r, Eppendorf Ibèrica, San Sebastián de los Reyes, Spain).

Frozen-thawed sperm processed by submerging the frozen straw in 1 mL of G-MOPS at 37ºC was used in all conditions. ICSI was performed using a 7 μm glass sharp micropipette (IC-50-30, Origio, CooperSurgicalFertility Companies, Målov, Denmak).

The oocyte was then subjected by the holding pipette, placing the polar body in 6-12 o'clock site, while the zona pellucida was removed using Piezzo Drill pulses. Before injection, a single motile spermatozoon with normal morphology was immobilized using the ICSI pipette. Once the sperm was aspirated into the pipette, it was immobilized using Piezo Drill pulses and then aspirated and moved to an awaiting droplet. When the sperm and the oocyte were prepared, the sperm was aspirated with the pipette again. Then, the pipette was inserted into the oocyte cytoplasm by breaking first the oolemma.

Figure 3: Donkey egg immediately before ICSI (image courtesy by Jordi Miró)

2.6 EMBRYO DEVELOPMENT

The future zygotes were cultured in a global medium (LGGG, LifeGlobal, Guilford, CT) with 10% v/v FBS for up to 11 day). At day 5 the media was renewed, and the cleavage was assessed.

Embryos were often evaluated from day 7 to day 11, removing them from the incubator for a microscopic evaluation. However, in some cases, embryos were assessed from time-lapse images. In these cases, presumptive ICSI zygotes were cultured using 16-microwell Primo Vision microwell culture dish (Vitrolife, Goteborg, Sweden) under 50 mL of culture media drop covered with mineral oil (ART-4008PA, Origio CooperSurgicalFertility Companies, Målov, Denmak). To control the development of the embryo, images were captured every 5 minutes starting 5-20 min after ICSI using the Primo Vision Time-Lapse System. The light used to take the images was changed according to each developmental stage, reducing it to less than what was considered potentially damaging. The image of each zygote was assessed evaluating the mitotic process, capturing images of each division until reaching the blastocyte stage. Assessing blastomere division allows us to determine the time of cleavage: when it is first visible the indentation of the oolemma. It also let to recognize blastocyst stage and to detect cell death when the image shows streaming of the cytoplasm, degeneration, or necrotic breakdown of the cells (Meyers et al., 2019). Blastocyst were vitrified for future transference into a reception mare or jenny or for future studies.

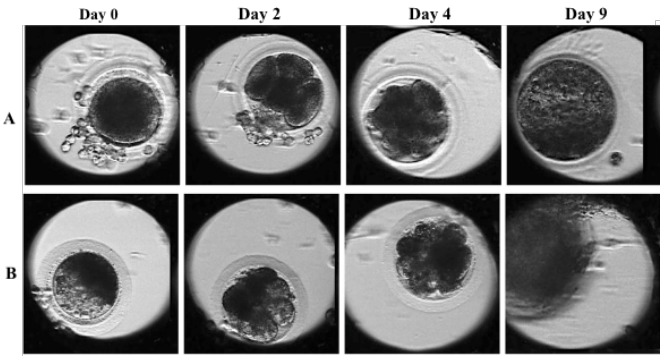

Figure 4: Time-lapse images of the development of donkey (A) and horse (B) embryos (image courtesy by Jordi Miró)

2.7 STATISTICAL ANALYSES

Statistical package R, using R-commander software, was used to analyse the results obtained in this work. Shapiro-Wilk and Levene tests were conducted to assess data distribution and

homogeneity of variances respectively. Following this, Student-t two-tailed test was performed for all data comparisons except for oocyte recuperation rate and blastocyst/injection oocyte as the first data in mares and the second in jennies do not follow a normal distribution. A Wilcoxon-Mann-Whitney two tailed test was performed in oocyte recuperation rate and a Brunner Munzel two tailed test for blastocyst/injection oocyte, as this parameter has unequal variances. In all analyses, the level of significance was set at $P \leqslant 0.05$. Data are shown as mean ± standard deviation (SD).

3. RESULTS

3.1 OVUM PICK UP IN MARES

OPU results are shown in Table 1. From a total of 287 aspirated follicles in 16 OPU sessions, only 5 sessions resulted in poor collection rates (under 53.0%). For mares 1, 2 and 7, more than one OPU was done. Variable number of COCs obtained are present. In mare 7, although two OPU sessions were conducted, no COCs were obtained so ICSI was not performed (11%). In the rest, at least more than 2 COCs were aspirated after OPU. On average, 12.31 oocytes per session were recovered with an average oocyte collection efficiency of 68.64 ± 30.06 %.

Table 1: Individual efficiency of OPU in mare

No. OPU/ICSI	Mare ID	No. Aspirated follicles	No. Oocytes recovered	Oocyte collection efficiency (%)
1	1	38	29	76.32
2	2	17	17	100.00
3	1	14	14	100.00
4	1	20	16	80.00
5	2	15	11	73.33
6	2	19	13	68.42
7	2	9	4	44.44
8	2	20	16	80.00
9	2	18	16	89.89
10	2	24	17	70.83
11	2	8	4	50.00
12	2	25	16	64.00
13	2	16	14	87.50
14	3	6	4	66.67
15	4	5	4	80.00
16	6	9	2	22.00
17	7	10		0.00
18	7	10		0.00

ID, identification number. No, number.
Oocyte collection efficiency was calculated as the no. oocytes recovered per no. of aspirated follicles.

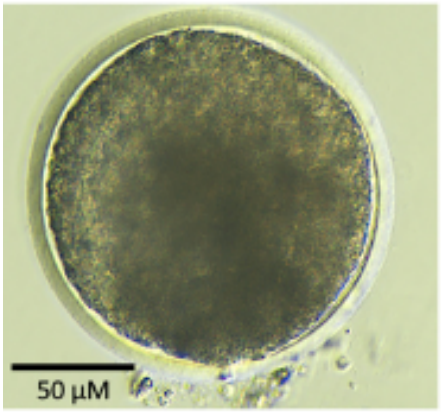

Figure 5: ICSI blastocyst from a horse (image courtesy by Jordi Miró)

3.2 *IN VITRO* PRODUCTION OF EMBRYOS IN MARES

ICSI individual results are showed in Table 2. At least a blastocyst was obtained in all cases, except for mare 7, where no oocyte was recovered, or mare 6. In case number 8 to 13, some information is missing due to communication with laboratories. Results from mares 7 and those with lacking data were not considered for the analyses. The overall results are summarized in Table 4.

Table 2: *Individual efficiency of transvaginal follicle aspiration and ICSI in mares*

No.	Mare ID	Stallion ID	No. injected oocytes	No. Cleaved zygotes	No. Blastocyst	Maturation rate (%)	Cleavage rate (%)	Blastocyst/ injected oocyte rate (%)
1	1	1	19	16	2	65.52	84.21	10.53
2	2	2	16	12	3	94.12	75.00	18.75
3	1	2	13	8	1	92.86	61.54	7.69
4	1	3	11	10	1	68.75	90.91	9.09
5	2	4	3	2	1	27.27	66.67	33.33
6	2	1	8	7	3	61.54	87.50	37.50
7	2	1	3	2	1	75.00	66.67	33.33
8	2	3			3			
9	2	2			2			
10	2	5			3			
11	2	6			1			50.00
12	2	3			5			50.00
13	2	2			3			
14	3	7	2	1	1	50.00	50.00	
15	4	8	4	4	2	100.00	100.00	
16	6	9	1	1	0	50.00	100.00	
17	7	10			0			
18	7	10			0			

ID: identification number. No: number. No. ICSI: oocytes that underwent ICSI.
Maturation rate was calculated as the no. of MII oocytes out of the total no. of oocytes collected. Cleavage rate was defined as the ratio of no. cleaved zygotes by the no. oocytes injected oocytes.

3.3 OVUM PICK UP IN JENNIES

Table 3:*Individual efficiency of transvaginal follicle aspiration in donkeys*

Jenny ID	No. Follicles aspirated	No. Oocytes recovered	Oocyte collection efficiency (%)
1	11	8	72.7
1	14	9	64.3
2	8	4	50.0
2	7	5	71.4
3	6	5	83.3
3	3	3	100.0
4	10	6	60.0
5	6	5	83.3

ID: identification number. No: number.
Oocyte collection efficiency was calculated as the no. oocytes recovered per no. of aspirated follicles.

Results in donkeys are summarised in Table 3 and 4. Sixty-five aspirations were done in five jennies and 45 oocytes were collected. The minimum number of COCs obtained was three and the maximum nine with an average of 5.63.

3.4. *IN VITRO* PRODUCTION OF EMBRYOS IN JENNIES

No.	No. injected oocytes	No. Cleaved	No. Blastocyst	Maturation rate (%)	Cleavage rate (%)	Blastocysts/ injected oocyte (%)
1	7	5	0	87.50	71.43	0.00
1	6	4	0	66.67	66.67	0.00
2	3	1	0	75.00	33.33	0.00
2	3	2	0	60.00	66.67	20.00
3	5	2	0	40.00	60.00	0.00
3	1	2	1	33.33	100.00	0.00
4	6	6	0	100.00	100.00	0.00
5	3	0	0	60.00	0.00	0.00

No:number.
Maturation rate was calculated as the no. of MII oocytes out of the total no. of oocytes collected. Cleavage rate was defined as the ratio of no. cleaved zygotes by the no. oocytes injected oocytes.

3.5. COMPARISON BETWEEN MARES AND JENNIES RESULTS

Table 4: *OPU average result rates in mares and jennies*

Group	No. Follicles aspirated	No. Oocytes recovered	Oocyte recuperation efficiency (%)	No. ICSI
Mares (n=8)	287	197	64.08± 30.06	80
Jennies (n=5)	65	45	73.13 ±15.66	34

Data are shown as mean ± SD. No: number.

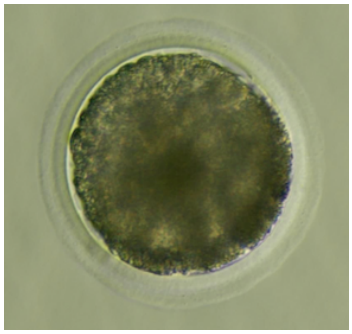

Figure 6: The only ICSI blastocyst from a jennie obtained to the date (image courtesy by Jordi Miró)

Table 5: *ICSI average result in mares and jennies*

Group	Maturation rate (%)	Cleavage rate (%)	No. obtained blastocysts	Blastocysts/ injected oocyte (%)
Mares	68.51±22.92	78.25± 16.94	32	27.80±16.87*
Jennies	65.31 ±22.40	62.26±33.14	1	2.50 ± 7.07*

The subscript (*) means significant differences (P≤ 0.05) between results in mares and jennies.
Data are shown as mean ± SD. No: number.

No significant differences were observed in oocyte recovery efficiency (P= 0.7414), oocyte maturation rate (P= 0.7705) or cleavage rate (P=0.202129), but there were significant differences in the final ratio of blastocyst/injected oocytes (P=0.00208).

4. DISCUSSION

The interest in OPU-ICSI commercial programs is increasing rapidly during the last years. For this reason, establishing efficient protocols for collecting, maturing and producing embryos from collected oocytes is needed. Results are still not promising, especially for ICSI. Results of 50 to 60% of picking up oocyte have been previously reported the last (Cuervo-Arango et al., 2019b; Claes et al., 2022; Rader et al., 2016; Barbacini et al., 2009). Our results raised up to 64.08% success in horse and 73.13% in donkeys, validating our technique. It is clear that the number of recovered oocytes can affect the success of the commercial OPU-ICSI program. This parameter is often affected by age, since the number of follicles aspirated is lower in mares above 20 years but, interestingly, the percentage of maturation seems to be significantly higher (Cuervo-Arango et al., 2019a). Furthermore, other parameters, which are not corroborated yet, could also explain the different findings in individual results per animal. In our study, any oocyte was collected by OPU from mare 7, perhaps because of an intrinsic reproductive problem. It is not strange that no embryo is obtained, since it is estimated that 35% of immature follicles aspirated will yield no embryo (Rader et al., 2016).

The serum level of anti-müllerian hormone, that reflects the number of follicles, was assessed as an outcome for success in OPU. However, large variations in circulation levels were observed (Papas et al., 2021) suggesting that assessment of follicle could be the main criteria for suitability of OPU-ICSI success as we did.

The highest percentage of oocyte recovery per aspirated follicles is obtained in non-breeding season, when most of the mares are in anoestrus, having small follicles which tend to lead to higher recovery efficiency because the needle can reach a greater proportion of the follicle surface wall (Cuervo-Arango et al., 2019b). Higher number of follicles per aspiration are reported when aspiration is timed according to follicle availability (Jacobson et al., 2010). On the other hand, lower number of follicles aspirated may be due to shorter time interval between two consecutive OPUs (Iacono et al., 2014). Taking this into account, more parameters, such as the date of the OPU session should be studied to know if, for example mare number 2 that had two OPUs with only 4 oocytes could have other with far more. Aspiration of follicles every 7 days or every 4 days was associated with significant decrease in average follicles number (Jacobson et al., 2010). Recovery of immature equine oocytes is complicated by the closer and stronger attachment of the equine oocyte-cumulus complex to the follicle wall (Hinrichs, 2010). Perhaps, the incorrect position of the needle inside the follicle combined with insufficient movement of the needle during the aspiration could also explain low oocyte recovery (Galli et al., 2018).

In our study, no hormone treatments were used for increasing the number of follicles. Although progesterone administration seems to have no effect on oocyte recovery in horses (Jacobson et al., 2010), the use of a low-dose FSH increases oocyte recovery but has no effect on oocyte morphology or development competence after ICSI (Altermatt et al., 2009).

The main limitation of the study is that we used two different ICSI laboratories, and both external, so slight changes such as the way of processing oocytes, spermatozoa and embryos may have affected the results. Nevertheless, the overall average of maturation of oocytes (68.5% in mares and 65.3 % in jennies) in our study were comparable to that obtained in other laboratories in mares (Rader et al., 2016; Barbacini et al., 2009; Claes et al., 2022). Hence, time of oocytes collection and duration of maturation make it difficult to compare maturation and developmental results among laboratories, or to compare composition of maturation media and developmental competence (Hinrichs, 2010)

Regarding the production of embryos, ICSI results vary greatly between studies, from 10.0-23.0% of average blastocysts/oocyte injected have been previously described (Meyers et al., 2019; Barbacini et al., 2009.; Rader et al., 2016; Cuervo-Arango et al., 2019b). Moreover, in individual cases it has been reported a 46.7% of success (Cuervo-Arango et al., 2019b). The same authors also described more than 167 blastocyst/OPU, the grater results published by the moment. Our study leads to a 27.80% of blastocysts/injected oocyte in horses, an average greater than any mentioned before. However, some poor individual results are present, having 5 ICSI with less than 10.0% blastocysts/injected oocyte. It is important to point out that the percentage of cleavage is 78.25% in mares and 62.26% in jennies. We suggest that *in vitro* culture of embryos is an important limitation and further research is required.

Several methods have been performed for blastocyst production. However, the development is sub-optimal in many cases (Matsukawa et al., 2007; Meyers et al., 2019; Merlo et al., 2018). It has been described that holding temperature and injection technique can affect these results. Nevertheless, another parameter to consider is the culture protocol after ICSI, which can influence the blastocyst development (Metcalf et al., 2020). Few studies have produced results that definitively describes the embryo media requirements during this development. However, when comparing with other research (Brom-de-Luna et al., 2021; Cuervo-Arango et al., 2019b), this may indicate that the percentage achieved on mares with the present culture system is near the maximum possible considering de knowledge of this moment. Some modifications of oocyte handling and maturation systems are promising (Galli et al., 2018; Meyers et al., 2019). Even though, bigger number of trials should be done to obtain more consistent results. Follicular fluid, which is the natural environment during the maturation *in vivo,* has been studied for development competence *in vitro*, showing similar results as when using FBS and proposing it to enhance oocyte fertilization after ICSI (Bùgh et al., 2002) but it is something not studied before in donkeys. It is noticeable that the only donkey blastocyst was achieved when eggs were maturated using a medium supplemented with preovulatory follicular fluid. Previous results and knowledge suggest that further studies of the metabolomic, proteomic and endocrine profile of follicular fluid and tests on which molecules can improve the maturation are needed, as it would be useful to create new medium that recreates the biological requisites for *in vivo* oocytes to improve the overall results.

Not only the age could explain lower percentage of blastocyst but also breeds; Arabian horse oocytes leads in poor results when compared with warmblood mares (Galli et al., 2016), separating and studying individual cases would confirm if this has influenced our results. Stallion identity appeared to have less effect on the efficiency of blastocyst production, even the sperm with the worst quality is able to produce a blastocyst using ICSI protocols. Even though male effects are described (Cuervo-Arango et al., 2019b), it is not relevant enough to consider as a possible factor

to consider. Furthermore, seven different sperm is used in a low number of mares, so it cannot be evaluated, and consequently it is not a parameter to change to improve the overall results.

In the present report, the mean number of oocytes recovered from jennies was 5.63, which is higher than the last study reported (Deleuze et al., 2018), but less than in mares, not only on average (10.98) but on the maximum obtained individually (9 and 19 respectively). However, similar percentages of recovery are obtained in both species, which validates that the OPU technique and reflects the skills of the operators have been easily transferred to donkeys. These findings substantiate that OPU procedure can be done in donkeys as performed in mares, with similar collection rates.

Similar oocyte maturation results are present in our study between the two species. The optimal conditions for oocyte culture in donkeys are still unknown. However, the use of culture media with 10% PFF, as other culture media did not work before, has demonstrated satisfactory results as some matured and finally became one blastocyst after ICSI. However, after ICSI, the results between mares and jennies started to differ more significantly with a percentage of cleavage of only 78.25% in horses compared with 62.26% in jennies and a final efficacy result of 27.80% and 2.50% respectively. This suggest that ICSI in donkeys might not be as efficient as in horse. It has been proposed that when evaluating embryo development by time-lapse imaging, donkey *in vitro* embryos have a similar cell division patter compared to the horse (Flores Bragulat et al., 2023) that means that probably improper *in vitro* maturation and culture conditions and/or incomplete donkey oocyte activation after ICSI by donkey sperm are the main conditioning to lead in poor developmental rates on donkey ICSI embryos. Thus, investigation of effects of oocyte maturation and embryo culture media and sperm processing, as well as knowledge of which components can affect them negatively could improve greatly the results, not only in jennies but also in horse *in vitro* production of blastocysts. Another interesting research area is co-culture of donkey and horse embryos, which should be done to discover if this could increase the developmental competence of jenny oocytes.

5. CONCLUSIONS

In conclusion, the overall results in this study are comparable to those reported elsewhere; the oocyte collection efficiency, oocyte maturation rate and blastocyst obtained/injected oocyte rate obtained in this study are greater than those reported before. Moreover, this is the first time that a donkey blastocyst is reported. While these findings suggest that this technique gives the opportunity to breed equine valuable individuals and breeds in risk of extinction, more ICSI cycles are needed to confirm this tendency. Horse protocols of OPU and oocyte incubation for maturation can be extrapolated to jennies. However, ICSI in donkeys is not as efficient as in mares. The preovulatory follicular fluid supports the *in vitro* maturation and the embryo development of donkeys, only using this supplementation, blastocyst formation was accomplished. The possibility to collect oocytes from alive jennies and produce *in vitro* donkey embryos gives a significant contribution to genetical preservation of species of interest, such as Catalan Donkey.

Overall, the OPU-ICSI procedure is a potential breeding technique in equine species. As the present results are still suboptimal and the cost of the procedure is high, more research is needed to increase the number of blastocysts obtained in horses and specially in jennies.

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