Revised

Establishment of conditions for ovum pick up and in vitro maturation of jennies oocytes towards the setting up of efficient in vitro fertilization and in vitro embryos culture procedures in donkey (Equus asinus)

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ABSTRACT

Most wild and domestic donkey breeds are currently endangered or threatened. Their preservation includes the creation of a Genome Resource Bank. Embryos cryopreservation

allows the preservation of genetics from both male and female and is the fastest method to restore a breed. Since embryo production in vivo is limited in equids, our objective was to establish conditions for in vitro production of embryos in donkey using ovum pick up (OPU), in vitro maturation (IVM), in vitro fertilization (IVF) and in vitro culture of zygotes. Donkey cumulus-oocyte complexes (COCs) were collected by transvaginal ultrasound-guided aspirations (OPU) in adult cyclic jennies and in vitro matured in TCM199 supplemented with fetal calf serum and epidermal growth factor for 24, 30, 34 or 38 hours. They were pre-incubated with oviductal fluid for 30 minutes, co-incubated with frozen-thawed donkey semen treated with procaine for 18 hours and cultured for 30 hours in DMEM-F12 supplemented with $NaHCO_3$, fetal calf serum and gentamycin. From the five OPU sessions, we collected 92 COCs in 193 follicles (48%) with an average of 4.2 COCs per jenny. All COCs were expanded after over 24 hours IVM. At collection, jennies oocytes contained a germinal vesicle. Metaphase 1 oocytes were observed after 30 hours IVM and 44% were in metaphase 2 after 34 hours IVM. In our conditions, IVM of donkey oocytes was slower than IVM of equine oocytes and optimal duration for donkey oocytes IVM may be 34 hours. Only 15% of jennies oocytes contained 2 pronuclei after co-incubation with donkey spermatozoa and none of them developed further after 48 hours post-IVF. Moreover, some parthenogenetic activation occurred. Thus, the treatment of donkey sperm with procaine may not be efficient for in vitro fertilization. In conclusion, we established for the first time conditions for ovum pick up in jennies with high recovery rates. We showed that in vitro maturation of jennies oocytes can produce 44% of metaphase 2 oocytes after 34 hours in culture and we described for the first time the chronology of in vitro maturation of donkey oocytes. Further studies are in progress to establish efficient conditions for in vitro fertilization and development of donkey zygotes.

INTRODUCTION

Most wild equids are currently endangered or threatened in the wild, and in particular the Asiatic wild ass and the African wild ass, as mentioned in the Red List of endangered animal species of the International Union for the Conservation of Nature (IUCN) [1]. Moreover, many domestic donkey breeds are vulnerable to extinction such as the Asinina de Miranda donkey in Portugal [2], the Martina Franca donkey in Italy [3,4], the Zamorano-Leonés donkey in Spain [5], the American Mammoth Jack donkey in the USA [6]. Several donkey breeds are even nearly extinct with fewer than 100 active breeding jennies, such as the ‘Grand Noir du Berry’ donkey or the ‘Normand’ donkey in France (communication from the Institut

Français du Cheval et de l'Equitation : http://statscheval.haras-nationaux.fr/core/zone_menus.php?zone=229&r=1316), etc.

The actions that are currently undertaken to preserve endangered donkey breeds include the creation of a Genome Resource Bank. Genome resource banking requires cryopreservation of semen, oocytes and/or embryos. Embryos cryopreservation allows the preservation of genetics from both male and female and is the fastest method to restore a breed [7].

In vivo donkey embryo production after natural mating or artificial insemination has been reported previously with an embryo recovery rate per cycle from 50 to 76% [8,9]. In equids, embryo production in vivo is limited, since experimental induction of multiple ovulations has a low efficiency [10] and routine induction of multiple ovulations is still ineffective [6]. To our knowledge, no efficient superovulation treatment is available in the donkey.

Providing the collection of several oocytes per ovary, embryo production in vitro would allow the production of several embryos per cycle that could withstand cryopreservation owing to their small size [11]. Thus, this is the choice technique for embryo production for preservation of genetics. In vitro production requires several steps. The first step is the collection of several immature oocytes on a donor female using ovum pick up. The ovum pick up technique has been widely used in the equine [11-21]. However, to our knowledge, ovum pick up has never been described in jennies. The second step is in vitro maturation of the oocytes. To date, only two reports about in vitro maturation of abattoir derived donkey oocytes have been published [22,23]. Moreover, the optimal duration of in vitro culture has not been evaluated. The third step is in vitro fertilization of oocytes. Several attempts to establish an efficient conventional in vitro fertilization (IVF) technique in the equine have been performed, but IVF rates remained quite low and IVF techniques were not repeatable [24-30]. Intracytoplasmic Sperm Injection (ICSI) has been widely adopted to generate horse embryos in vitro, both for scientific purposes and in the horse breeding industry [11,31]. However, ICSI requires expensive equipment and expertise in micromanipulation. In 2009, a 60% rate of equine IVF was reported after treatment of fresh spermatozoa with procaine [32]. However, procaine was reported to induce cytokinesis in horse oocytes [33]. To our knowledge, this IVF technique has never been tested in the donkey. The last step for in vitro embryo production is in vitro culture of the embryo to a developmental stage that allows cryopreservation. Up to now, no study has been published concerning in vitro culture and development of donkey embryos. Thus, there is a huge lack of knowledge concerning ovum pick up, in vitro maturation of oocytes, in vitro fertilization and in vitro development of embryos in the donkey.

Since there is an urgent need of techniques for in vitro embryo production for the preservation of endangered donkey breeds, our objective was to establish conditions for ovum pick up, in vitro maturation, in vitro fertilization and in vitro development of embryos in donkeys.
To ascertain that our in vitro conditions are appropriate for oocyte maturation and fertilization, equine oocytes were used as a control.

MATERIALS AND METHODS

All procedures on animals were conducted in accordance with the guidelines for the care and use of laboratory animals issued by the French Ministry of Agriculture and with the approval of the ethical review committee (Comité d'Ethique en Expérimentation Animale Val de Loire) under number 02701.01.
All chemicals were purchased from Sigma-Aldrich (St Quentin Fallavier, France) unless otherwise indicated.

Collection of donkey immature oocytes

Donkey immature cumulus-oocyte complexes (COCs) were collected during the breeding season by transvaginal ultrasound-guided aspiration (ovum pick up: OPU) in the nine adult cyclic jennies from our experimental studfarm. Ovarian activity was assessed by routine rectal ultrasound scanning to choose jennies with several growing follicles from 5 to 25mm. Follicles were punctured by transvaginal ultrasound-guided aspiration with a double-lumen needle (length 700mm, outer diameter 2.3mm, internal diameter 1.35mm, Casmed, Cheam, Surrey, England) and a sectorial probe (Aloka SSD900) as previously described [14]. After follicular fluid aspiration, the follicle was flushed with PBS (Phosphate Buffered Saline, Dulbecco A, Oxoid, Basingstoke, Hampshire, England) and heparin (Choay, Sanofi Aventis 5 000 IU/ml) at 38°C. All aspirated fluids were examined for oocyte recovery and oocytes denuded of cumulus or degenerated oocytes showing shrunken, dense or fragmented cytoplasm, were discarded. During the collection procedure, jennies were injected detomidine (Medesedan®, 0.25ml/animal i.v., 10mg/ml detomidine, Centravet, Plancoet, France) and butorphanol (Dolorex®, 0.6ml/animal i.v., 10mg/ml butorphanol tartrate and 0,1mg/ml benzethonium chlorure, Centravet) for sedation and analgesia, dipyrone and butylscopolamine (Estocelan®, 30ml/animal i.v., 4mg/ml butylscopolamine, Centravet) for analgesia and antispasmodia. After puncture, jennies received a preventive antibiotic injection (Depocilline, 20ml/animal i.m., benzylpenicillin 170.41mg/ml, Intervet, Beaucouze, France).

Collection of horse immature oocytes

Equine immature COCs were collected during the breeding season from slaughtered mares in commercial abattoirs. Ovaries from females of unknown reproductive history were obtained at local commercial abattoirs immediately after females were killed. They were transported to the laboratory within 2 hours in 0.9% (w/v) NaCl at 32-38°C. COCs were collected using the aspiration procedure previously described by Goudet and collaborators [34]. Briefly, the tunica albuginea was removed and all follicles larger than 5mm were aspirated with an 18 gauge needle at 100mm Hg of vacuum pressure, then the ovaries were cut into thick sections with a scalpel blade to find other follicles within the ovarian stroma. Follicular fluids were examined under a stereomicroscope for COCs recovery. Oocytes denuded of cumulus and degenerated oocytes showing shrunken, dense or fragmented cytoplasm, were discarded. To avoid contaminations, COCs were washed in a medium containing antibiotics (Medium 199 with Earle's Salts, 25mM HEPES and $NaHCO_3$ supplemented with 20% (v/v) Fetal Calf Serum (FCS) and 25µg/ml gentamycin).

In vitro maturation (IVM) of horse and donkey immature oocytes

Donkey and horse COCs were washed in maturation medium: Medium 199 with Earle's salts supplemented with 20% (v/v) FCS and 50ng/ml Epidermal Growth Factor (EGF) [34]. They were cultured in groups of 10 to 30 in 500µl of maturation medium in an atmosphere of 5% CO_2 in air at 38.5°C in 100% humidity. Jennies COCs were cultured for 24, 30, 34 or 38 hours. Equine COCs were cultured for 24 or 30 hours.

Donkey semen collection, freezing and evaluation

The study included two mature donkeys (Grand Noir du Berry) of proven fertility, aged 7 and 14 years. The donkeys were housed at INRA in Nouzilly, France. Semen was collected on a regular basis (three times a week) with a closed artificial vagina (INRA model). After collection, raw semen was filtered through gauze to remove the gel fraction of the ejaculate and immediately frozen in INRA Freeze® extender according to the procedure described by Pillet and collaborators [35]. The straws were stored in liquid nitrogen until used.

Donkey semen evaluation was performed as follows. Three straws per ejaculate and per donkey were thawed in a water bath for 30 seconds at 37°C and diluted in INRA96® extender at 20 x 10^6 spermatozoa/ml. The diluted sperm cells were incubated at 37°C for 10 min and motility parameters evaluated using computer-assisted analysis (IVOS, version 10, Hamilton Thorne, Beverly, MA, USA). The parameters analysed were average path velocity (VAP,

µm.s-1), percent progressive sperm cells (PROG, average path velocity higher than 40 µm.s-1 and straightness of track higher than 80 %), and percent rapid sperm cells (RAPID, average path velocity higher than 40 µm.s-1). Analyses were performed in duplicate (2 x 3 µL sampling/straw) on three fields analysed (i.e. 6 observations/straw and 18 observations /donkey/ejaculate). The motility parameters of the 2 ejaculates used for IVF (1 per donkey) were respectively: VAP 121±0.7µm/sec and 96±4.3µm/sec; RAPID 67±3.4 and 69±1.8%; PROG 51± 4.4% and 47±2.4%.

In vitro fertilization (IVF) procedure

Pre-incubation of oocytes with oviductal fluid

After 27 hours in vitro maturation, COCs were partially denuded by flushing and washed in modified Whitten's Medium (MW; 100mM NaCl, 4.7mM KCl, 1.2mM $MgCl_2$, 22mM Hepes, 4.8mM lactic acid hemicalcium salt, 1mM pyruvic acid) supplemented with 5.5mM glucose (anhydrous), 25mM $NaHCO_3$ and 7mg/ml BSA, pH 7.25 [32].

COCs were then pre-incubated in droplets of 30µl of porcine oviductal fluid for 30 minutes in an atmosphere of 5% CO_2 in air at 38.5°C in 100% humidity, as previously described [36]. For porcine oviductal fluid collection, genital tracts from gilts with both ovaries containing several follicles larger than 5mm were used. They were obtained at a commercial abattoir and transported to the laboratory at room temperature. The oviducts were dissected free from surrounding tissues. The oviductal content from the ampulla was expelled by gentle squeezing using a sterile microscope slide and collected by introducing the tip of a pipette into the ampulla and aspirating while making a manual ascendant pressure from the isthmus to the ampulla [37]. After centrifugation at 10 000g for 15min, the supernatant containing secreted and intracellular components was immediately stored at -20°C until use as "oviductal fluid".

Preparation of donkey jack sperm and in vitro fertilization

Frozen donkey semen (100 X 10^6/ml) from a single ejaculate of two jacks (2 straws per jack) was thawed at 37°C for 30 sec and pooled. Then, 2ml of semen was diluted in 2ml of pre-warmed MW supplemented with 5.5mM glucose (anhydrous), pH 7.25 [32]. Diluted sperm was transported to the laboratory within a few minutes at 37°C and centrifuged in 15ml conical tubes at 100g for 1min at 37°C to remove particulate matter and dead sperm. The supernatant was then transferred to a 14ml round bottom tube and centrifuged at 600g for 5min at 37°C. The pellet was re-suspended in 1.5ml of pre-warmed MW supplemented with glucose, and the concentration was determined by counting on a Thoma chamber under a

microscope (Olympus, IMT-2, Paris, France). Spermatozoa were then diluted at 10 x 10^6/ml in pre-warmed MW supplemented with 5.5mM glucose, 25mM $NaHCO_3$ and 7mg/ml BSA, pH 7.25 (capacitating MW) [32]. Spermatozoa were incubated in 500µl aliquots in polyvinyl alcohol-coated 5ml round-bottom tubes at 37°C in a humidified atmosphere during 6 hours. The motility was visually evaluated under a microscope (Olympus, IMT-2, Paris, France) at the beginning and at the end of the incubation period. Spermatozoa were then diluted at 5 x 10^6/ml in capacitating MW supplemented with 5mM procaine to induce hyperactivated motility. Droplets of 100µl of spermatozoa suspension were laid down onto culture dishes and covered with mineral oil.

After pre-incubation with oviductal fluid, horse and donkey oocytes were washed in capacitating MW and groups of 10 were transferred to droplets of 100µl of donkey spermatozoa suspension in capacitating MW supplemented with 5mM procaine. Oocytes were co-incubated for 18 hours in an atmosphere of 5% CO_2 in air at 38.5°C in 100% humidity.

For the control group without spermatozoa, horse and donkey oocytes were transferred to droplets of 100µl of capacitating MW supplemented with 5mM procaine without donkey spermatozoa. They were incubated for 18 hours in an atmosphere of 5% CO_2 in air at 38.5°C in 100% humidity.

In vitro culture of zygotes

After 18h incubation with or without spermatozoa, zygotes were washed and flushed to remove attached spermatozoa in DMEM-F12 with L-glutamine and Hepes (ref D8900) supplemented with 1.2g/l $NaHCO_3$, 10% FCS and 25µg/ml gentamycin (culture medium). Groups of 10 were transferred to droplets of 30µl of culture medium for 30h (48h post-IVF) in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.5°C in 100% humidity.

Assessment of nuclear status

Nuclear status was assessed either after 0, 24, 30, 34 or 38h in vitro maturation or 48h post-IVF. Oocytes and zygotes were washed by aspiration in and out of a pipette in PBS, fixed in 4% paraformaldehyde in PBS for 20min at room temperature, washed in PBS and processed for analysis.

After in vitro maturation, only DNA staining was performed. Oocytes were incubated with 1µg/ml bis-benzimide (Hoechst 33342) in PBS for 5 minutes and mounted on microscope slides in Mowiol V4-88 (133mg/ml; Hoechst, Frankfurt, Germany) and n-propyl gallate (5mg/ml).

DNA and nuclear membrane staining were performed on zygotes 48h post-IVF. They were incubated for 30min at room temperature in 0.2% Triton X-100 in PBS. Nonspecific reactions were blocked by incubation for 1 hour at room temperature in 10% goat serum in PBS. Oocytes and zygotes were incubated overnight at 4°C or 2 hours at room temperature with an anti-lamin A/C antibody (Thermo scientific) diluted 1:100 in PBS containing 0.2% BSA and 0.1% Tween. After 4 washing for 5min in PBS containing 0.2% BSA and 0.1% Tween, they were incubated for 1 hour at room temperature with an AlexaFluor 594-conjugated-anti-mouse antibody diluted 1:400 in PBS. They were then washed 5 times for 5min in PBS containing 0.1% Tween and 2 times for 5min in PBS. They were incubated with 1µg/ml bis-benzimide (Hoechst 33342) in PBS for 5 minutes and mounted on microscope slides in Mowiol V4-88 (133mg/ml; Hoechst, Frankfurt, Germany) and n-propyl gallate (5mg/ml). The slides were kept at 4°C in darkness until observation under an epifluorescence microscope (Zeiss). Controls were performed using no primary antibodies to ascertain the absence of non-specific binding or no secondary antibodies to ascertain the absence of auto-fluorescence.

Statistical analysis

The percentages of mature oocytes were compared using chi-square analysis. The percentages of oocytes with 2 pronuclei were compared using Yates' chi-square analysis. Differences were considered statistically significant at $p<0.05$.

RESULTS

Oocyte recovery

For jennies oocytes collection, five puncture session were performed on adult cyclic jennies during follicular phase (ovaries containing growing follicles without corpus luteum). From the 5 puncture sessions, 193 follicles were flushed and 92 COCs were collected (48%) with an increase of the recovery rate along the puncture sessions from 34% to 56% (Table 1). Globally, we collected an average of 4.2 COCs per female, with a maximum of 10 COCs per female in 2 jennies.

For equine oocytes collection, a total of 28 ovaries were recovered in a local slaughterhouse and 67 oocytes were collected (2.4 oocyte/ovary).

Cumulus aspect 0 to 38h post-IVM

At collection, 4 donkey COCs were expanded and 88 were compact (96%; Figure 1a). All

donkey COCs were expanded after over 24 hours in vitro maturation (Figure 1b).
At collection, 4 equine COCs were expanded and 63 were compact (94%). All equine COCs were expanded after over 24 hours in vitro maturation.

Nuclear stage 0 to 38h post-IVM

Nuclear stage of jennies oocytes at collection (0h) or after 24, 30, 34 or 38 hours IVM are presented in table 2. At collection (0h post-IVM), 14 jennies oocytes were analysed. Most of them contained a germinal vesicle, either with distinct chromatin filaments (6/14; filamentous germinal vesicle in table 2; figure 1c) or with chromatin partly condensed (7/14; partly condensed germinal vesicle in table 2; figure 1d). Nine jennies oocytes were analysed after 24 hours IVM. Most of them contained a germinal vesicle, either with distinct chromatin filaments (4/9; figure 1c) or with chromatin partly condensed (2/9; figure 1d), two of them contained condensed chromatin (figure 1e). Nine jennies oocytes were analysed after 30 hours IVM. Four of them contained a germinal vesicle with distinct chromatin filaments (figure 1c) and four of them contained a metaphase 1 (figure 1f). Nine jennies oocytes were analysed after 34 hours IVM. Four of them contained a metaphase 2 with a polar body (figure 1g). Eight oocytes were analysed after 38 hours IVM. Two of them contained a metaphase 2 starting to decondense (figure 1h), two contained condensed chromatin and two contained partly condensed germinal vesicle.
Equine oocytes were analysed after 24 hours (n=6) or 30 hours (n=6) IVM (Table 3). After 24 hours IVM, half of the equine oocytes were in metaphase 2. After 30 hours IVM, four out of six of the equine oocytes were in metaphase 2, the two other being degenerated.

Nuclear stage 48 hours post-IVF

Oocytes from donkey jennies and oocytes from pony mares were co-incubated with or without donkey sperm and analysed 48 hours later (Table 4). They contained condensed chromatin (figure 1e), metaphase 2 (figure 1g), one pronucleus (figure 1i, 1j) or two pronuclei (figure 1k, 1l). The percentage of mature oocytes (metaphase II + 1 pronucleus + 2 pronuclei) was not significantly different between donkey (34/42, 81%) and equine (37/55, 67%) oocytes (chi-squared test, $p>0.05$). The percentage of oocytes containing 2 pronuclei after incubation with donkey sperm was not significantly different between donkey (15%) and equine (21%) (Yates' chi-squared test, $p>0.05$). The percentage of oocytes containing 2 pronuclei was not significantly different after incubation with vs without sperm in the donkey (15% vs 6%) or in the equine (21% vs 11%) (Yates' chi-squared test, $p>0.05$).

DISCUSSION

Most wild donkeys are currently endangered or threatened and many domestic donkey breeds are at risk of extinction. Embryos cryopreservation allows the preservation of genetics from both male and female and is the fastest method to restore a breed [7]. Thus, there is an urgent need of techniques for in vitro embryo production for the preservation of endangered donkey breeds. Our objective was to establish conditions for ovum pick up, in vitro maturation, in vitro fertilization and in vitro development of embryos in donkey.

Early attempts at in vivo collection of equine oocytes were made via standing flank transcutaneous puncture [38,39]. Then, transvaginal ultrasound guided follicular puncture was developed in the mare [40,41]. Since then, the ovum pick up technique has been extensively used in the equine [11-21]. The mean number of follicles punctured per mare per session ranges from 2 to 12 with an effect of mare [19], breed (lower number in pony mares compared to saddle mares) [12], the estrous cycle stage [14,15] and season [17]. The mean number of immature oocytes recovered per mare per aspiration session ranges from 0 to 5 and the oocyte recovery rate per immature follicle in the mare range from 20% to 56% [12-15,17-19]. In our study, the mean number of immature oocytes recovered from jennies ranged from 2.75 at the first session to a maximum of 6 at the forth session. The oocyte recovery rate per immature follicle in the jennies ranged from 34% at the first session to 56% at the fifth session. Since the operators that performed ovum pick up in jennies in our study were used to perform ovum pick up in the mare with high recovery rates, this could indicate that a couple of sessions are necessary to adapt to jennies. However, recovery rates of immature oocytes from jennies similar to recovery rates from mares were quickly reached. In our hands, transvaginal ultrasound guided puncture of donkey immature follicles is feasible and can reach in some attempts a 56% recovery rate and a mean number of 6 oocytes per female.

To date, only two reports about in vitro maturation of abattoir derived donkey oocytes have been published to the best of our knowledge [22,23]. In 2011, Zhao and collaborators used TCM199 or DMEM-F12 supplemented with fetal calf serum, porcine FSH, equine LH, insulin-like growth factor, insulin-transferrin-selenium, taurine, L-cysteine, L-glutamine, sodium pyruvate and gentamycin. The percentage of metaphase 2 was 55% for compact COCs cultured for 30-36 hours and 77% for expanded COCs cultured for 24-30 hours, with no significant differences between media [22]. In 2014, Abdoon and collaborators used DMEM with high glucose, DMEM with low glucose, DMEM-F12, TCM199, TCM199-F12 or CR1aa medium supplemented with fetal calf serum, FSH, hCG and gentamicin, the

percentage of metaphase 2 after 36 hours of culture was 41%, 39%, 46%, 56%, 69%, 62%, respectively [23]. This demonstrates that TCM199 seems to be an efficient basal medium for jennies oocyte maturation. In our study, in vitro maturation of jennies oocytes was performed in TCM199 supplemented with fetal calf serum and epidermal growth factor. This medium has been used in the equine with high maturation rates (64% [34], 65-74% [42], 73-76% [43], 71% [18], 61% [36], and 67% in this study). In the present work, this medium was efficient to sustain cumulus expansion in donkey oocytes since all donkey COCs were expanded after over 24 hours in vitro maturation. The optimal duration of in vitro culture has never been evaluated in donkey oocytes. Therefore, we analysed the chronology of in vitro maturation from 0 to 38 hours in culture. At collection, jennies oocytes contained a germinal vesicle, either with distinct chromatin filaments or with chromatin partly condensed. These meiotic stages were observed in equine oocytes immediately after in vivo collection [44,45]. After 24 hours in vitro maturation, some jennies oocytes contained condensed chromatin, and metaphase 1 oocytes were observed after 30 hours. In the equine oocytes, metaphase 1 were observed after 24 hours in vitro maturation which agrees with previous papers reporting that 25 to 37% of the equine oocytes are in metaphase 1 after 24 hours of in vitro maturation [45,46]. It appears that, in our conditions, equine oocytes seem to reach the metaphase 1 stage faster than donkey oocytes. About half of the donkey oocytes were in metaphase 2 after 34 hours in vitro maturation in our conditions, whereas half of equine oocytes were in metaphase 2 after only 24 hours, which is consistent with previous papers reporting that 25 to 35% of the equine oocytes are in metaphase 2 after 24 hours in vitro maturation [45,46]. In our conditions, in vitro maturation of oocytes from jennies seems to be slower than that of equine oocytes. Finally, after 38 hours in vitro maturation, 25% of donkey oocytes contained metaphase 2 starting to decondense and 25% were degenerated. Based on our observation, we suggest that the optimal duration for in vitro maturation of donkey oocytes should be 34 hours. Our data are consistent with the results from Zhao and collaborators who obtained 55% of metaphase 2 oocytes from jennies after 30-36 hours of in vitro maturation [22]. Abdoon and collaborators obtained higher in vitro maturation rates with donkey oocytes after 36 hours of culture using TCM199-F12 or CR1aa medium supplemented with fetal calf serum, FSH, hCG and gentamicin (69% and 62%) [23]. Thus, our in vitro maturation medium, though efficient for equine oocytes, may not be optimal for donkey oocytes.

After in vitro maturation, jennies oocytes were pre-incubated with oviductal fluid. Pre-incubation of equine oocytes with oviductal fluid or oviductal cells significantly increases the fertilization rate after treatment of sperm with calcium ionophore [28] or with procaine [36].

Based on our previously published review on the beneficial effect of oviductal cells and secretions on equine in vitro fertilization [47], we anticipated a beneficial effect of pre-incubation of jennies oocytes with oviductal fluid. Jennies oocytes were co-incubated with donkey sperm treated with procaine, as previously described for equine gametes [32]. Equine oocytes were used as control. A 60% rate of equine IVF was reported after treatment of equine spermatozoa with procaine [32,36]. However, in our study, only 15% of donkey oocytes and 21% of equine oocytes contained 2 pronuclei after co-incubation with donkey spermatozoa treated with procaine. None of them developed further after 48 hours post-IVF. Thus, the treatment of donkey sperm with procaine may not be efficient for in vitro fertilization. Moreover, when donkey or equine oocytes were co-incubated without spermatozoa, 6% and 11% respectively contained 2 pronuclei, showing that some parthenogenetic activation occurs. Procaine has been shown to induce cytokinesis in equine oocytes [33]. Our study demonstrates that procaine induces parthenogenesis/cytokinesis in jennies oocytes.

Finally, jennies and equine zygotes were cultured in DMEM-F12 medium and none of them developed to embryos. However, it is difficult to conclude on the efficiency of DMEM-F12 medium for in vitro culture of jennies zygotes as the IVF technique likely interferes with the original quality of zygotes. DMEM-F12 medium has been used previously for in vitro culture of equine zygotes after ICSI with high cleavage rates [31,48,49]. One can speculate that this medium might be efficient for the culture of donkey zygotes as well, but this remains to be shown.

In conclusion, we established for the first time conditions for ovum pick in jennies with high recovery rates. We showed that in vitro maturation of jennies oocytes can produce 44% of metaphase 2 in our conditions. We described for the first time the chronology of in vitro maturation of donkey oocytes and showed that they appear to require more time to achieve their maturation than the equine ones. Further studies are in progress to establish efficient conditions for in vitro fertilization and development of donkey zygotes. Our study is an important contribution to the development of in vitro techniques for donkey embryo production. This is of major importance for preservation of genetics from wild and domestic endangered donkey breeds.

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555 **FIGURE LEGEND**

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557 Figure 1. a) Donkey COCs with a compact cumulus, x50; b) donkey COCs with an expanded
558 cumulus; x50; c) donkey oocyte containing a germinal vesicle with distinct chromatin
559 filaments stained with Hoechst (a cumulus cell is shown by the arrow); x400; d) donkey
560 oocyte containing a germinal vesicle with chromatin partly condensed stained with Hoechst;
561 x400; e) donkey oocyte containing condensed chromatin stained with Hoechst (two cumulus
562 cells are shown by the arrow); x400; f) donkey oocyte containing a metaphase 1 stained with
563 Hoechst; x400; g) donkey oocyte containing a metaphase 2 with a polar body (*) stained with
564 Hoechst; x400; h) donkey oocyte containing a metaphase 2 decondensing stained with
565 Hoechst; x400; i) donkey oocyte containing a pronucleus stained with Hoechst; x400; j)
566 donkey oocyte containing a pronucleus stained with anti-lamin antibodies; x400; k) donkey
567 oocyte containing two pronuclei stained with Hoechst; x400; l) donkey oocyte containing two
568 pronuclei stained with anti-lamin antibodies; x400.

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