Journal of Equine Veterinary Science 35 (2015) 869–872

Contents lists available at ScienceDirect

Journal of Equine Veterinary Science

journal homepage: www.j-evs.com

Short Communication

Evidences of Regular Estrous Cycles in Mules and Successful Use of These Animals as Recipients for Donkey Embryos

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ARTICLE INFO

Article history:
Received 28 May 2015
Received in revised form 7 July 2015
Accepted 22 July 2015
Available online 30 July 2015

Keywords:
Mule
Corpus luteum
Cyclicity
Follicle
Embryo transfer

ABSTRACT

Mules are hybrid females, commonly considered as infertile, resulting from a cross between a donkey and a mare. Few recent studies address the particularities of the reproductive system of the mules. Here, we present data showing consistent evidences of regular cyclicity in mules and also the first report of two births of donkey foals after embryo transfer into mule recipients with spontaneous estrous cycle. Ovaries of mules (n = 72) were obtained at a local slaughterhouse. Clear evidences of cyclicity were found in 61.1% (44/72) of the ovaries, being 45.8% (33/72) corpora lutea and 15.3 (11/72) large antral follicles. After follicle aspiration, we obtained oocytes with regular distribution of granulosa cells and homogenous characterization of ooplasm. Because of the evidence of cyclicity in mules, two embryos collected from a donkey were transferred into two mules with spontaneous estrous cycles. Eutocic births of donkey foals occurred after 372 and 379 days of gestation. In contrast to the classical concept of infertile females, we found clear evidences that a higher percentage of mules can present ovarian activity and these animals may be considered for reproductive biotechnologies, such as embryo recipients.
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1. Introduction

Mules (*Equus mulus mulus*) are hybrids resulting from a cross between a male donkey (*Equus asinus*) and a mare (*Equus caballus*). Female mules usually present desirable husbandry patterns such as resistance, adaptation to several weather, all associated with a comfortable riding [1,2]. However, there is little information on ovarian activity and the reproductive aspects of mules.

The presence of follicles and corpora lutea in the ovaries of mules was described many years ago [3,4]. However, there is a lack of information about the proportion of regular estrous cycles in these animals, which are classically considered as infertile [5,6].

Considering the potential use of mules in the reproductive biotechnologies, we investigated reproductive patters in this species, such as (1) the proportion of large follicles and corpora lutea; (2) the morphology of oocytes, and (3) the use of cyclic mules as recipients of donkey embryos.

2. Materials and Methods

2.1. Ovaries

During the reproductive season of horses in the southern hemisphere (latitude 23° 17′ 34″ S and longitude 51° 10′ 24″ W), mule ovaries (n = 72) were obtained from a local slaughterhouse, 40 km away from the laboratory (approximately 30 minutes of transport). The transport was carried out in a thermal container at room temperature, 20°C. Using scissors, tweezers and a scalpel, the ligaments, and connective tissues of the ovaries were removed and

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http://dx.doi.org/10.1016/j.jevs.2015.07.010

structures were inspected for the presence of antral follicles and corpora lutea. The follicles were aspirated with a 40 × 12 mm needle (18 G) connected to a 20-mL syringe. The follicular fluid was assessed for the presence of oocytes using a stereomicroscope (Nikon, Tokyo, Japan).

2.2. Histological Assessment of the Ovaries

Mule ovaries (n = 3) were sectioned into two hemiovaries (1.5 cm × 1 cm), fixed in Bouin's solution for 24 hours and kept in 70% alcohol prior the slides preparation. The samples were dehydrated in increasing concentrations of alcohol, diaphonized in xylene, and embedded in paraffin. Serial sections of 5-μm thick were generated using a rotary microtome (Leica, Wetzlar, Germany), and every 50th histological section was mounted onto a slide. Specimens were stained with periodic acid Schiff and hematoxylin. The slides were evaluated under a light microscope (Nikon, Tokyo, Japan).

2.3. Female Mules as Recipients of Donkey Embryos

Based on the occurrence of follicles, oocytes, and corpora lutea in the ovaries of mules, we consider the viability to transfer embryos into these animals. Two mules four years old with regular cycles were subjected to follicular control when a donor donkey was detected in estrus. When the donor donkey presented preovulatory follicles of 38 mm in diameter and grade 4 uterine edema (scales 1–4; [7]), the induction of ovulation was conducted using human chorionic gonadotropins IM (hCG; Vetecor, 2500 IU Hertape-Calier, Spain). At the next day, the donkey was subjected to natural mating using a donkey. Ovulation was identified by ultrasonography 48 hours after ride (D0 = day of ovulation). At D8.5, embryos were collected using the transcervical approach and a closed system with approximately 1 L of lactated ringer's solution. Each recovered embryo was washed in 10 drops of 100 μL of Holding plus (Embriolife, Vitrocell, Brazil) and transferred to a recipient mule, which showed signs of estrus up to 2 days after the donor. The recipient mules were subjected to the induction of ovulation using hCG 2500 IU, IM, after ultrasound identification of a follicle of 37 mm in diameter and grade 3 uterine edema. Ovulation was confirmed by ultrasonography (D0 = day of ovulation). On the day of embryo transfer (D5), there was a rigid uterine tone and corpus luteum of approximately 30 mm. The embryo transfer into the uterine body was performed after the same approach for mares but aided by transrectal palpation, due to the difficulty in passing the embryo device through the mule cervix, considering its small diameter.

The pregnancies were identified at day 16 and confirmed at days 25 and 160 by transrectal ultrasonography.

3. Results

The ovaries of mules were elongated, rounded, and kidney-shaped, with weight on average of 8.77 g. Regarding ovarian structures, signs of cyclicity were found in 61.1% of the ovaries (44/72) being corpora lutea in 45.83% (33/72) and large antral follicles in 15.27% (Figs. 1A and B). The antral follicles were successfully aspirated, with 11 oocytes from 11 follicles (100% of recovery rate; Fig. 1D). Three oocytes were classified as grade 1 (homogeneous ooplasm and intact layers of granulosa cells surrounding the zona pellucid). The remaining eight oocytes were classified as atretic: picnotic nuclei, heterogeneous ooplasm, and absence (total or partial) of granulosa layers.

The histological procedure was prepared with one slide by 50 sections (5 μm) at the microtome. A total of 174 slides were obtained, on which we observed preantral (primordial and primary) follicles in small clusters. The morphology of the mule follicles was similar to those described in equine ovaries [8], as shown at (Fig. 1C). Based on the evidence of cyclicity in mules, the transfer of two donkey embryos into two mules with regular estrous cycles and we obtained eutocic deliveries of donkey foals after 372 and 379 days (Fig. 1E and F).

4. Discussion

According to our knowledge, this is the first report describing birth of donkeys from cyclic mules used as embryo recipient. Also, we evaluated data from more than 70 mule ovaries, the largest number at the literature. We found strong evidences of regular cyclicity in 61.1% (44/72) of the mules, being 45.8 (33/72) with corpora lutea and 15.3 (11/72) with large antral follicles, from which we obtained 11 oocytes after aspiration.

The presence of follicles and corpora lutea in mules has been questioned due to the classical concept of infertility [5]. This concept is barely supported because on the lack of information at the literature, a consequence of the difficulty to obtain ovaries from these animals. The evidence of cyclicity was proposed long time ago [3], and follicles and corpora lutea were identified in ovaries of fetal mules [9]. However, all these articles presented data from few or nonidentified number of mules, which makes impossible to realize the proportion of cyclic animal. In the present report, we could evaluate a consistent number of ovaries and this aspect certainly provides a better understanding on the matter.

The presence of oocytes in mule follicles was first described by Taylor and Short [9]. However, these authors only reported degenerated oocytes. Previous studies in hinnies showed the degeneration of germinative cells starting at the moment of chromosomal paring. For females mules, the follicle development is not unexpected. However, with the progression of meiosis, after germinal vesicle break down, some oocytes and follicles would start a short development and subsequent degeneration [10–12]. After aspiration of 11 follicles, we obtained 11 oocytes, being three of them classified as grade 1, an important contribution for future works on oocyte competence in these species.

The two cyclic mules used as recipients were easily identified in estrus because they presented characteristic behavior of estrus, which were more closer to asinine species for example chewing movements, frequent urination in approaching male, and incisive demand for male.

The application of equine embryos transferred into recipient mules has been previously described but only

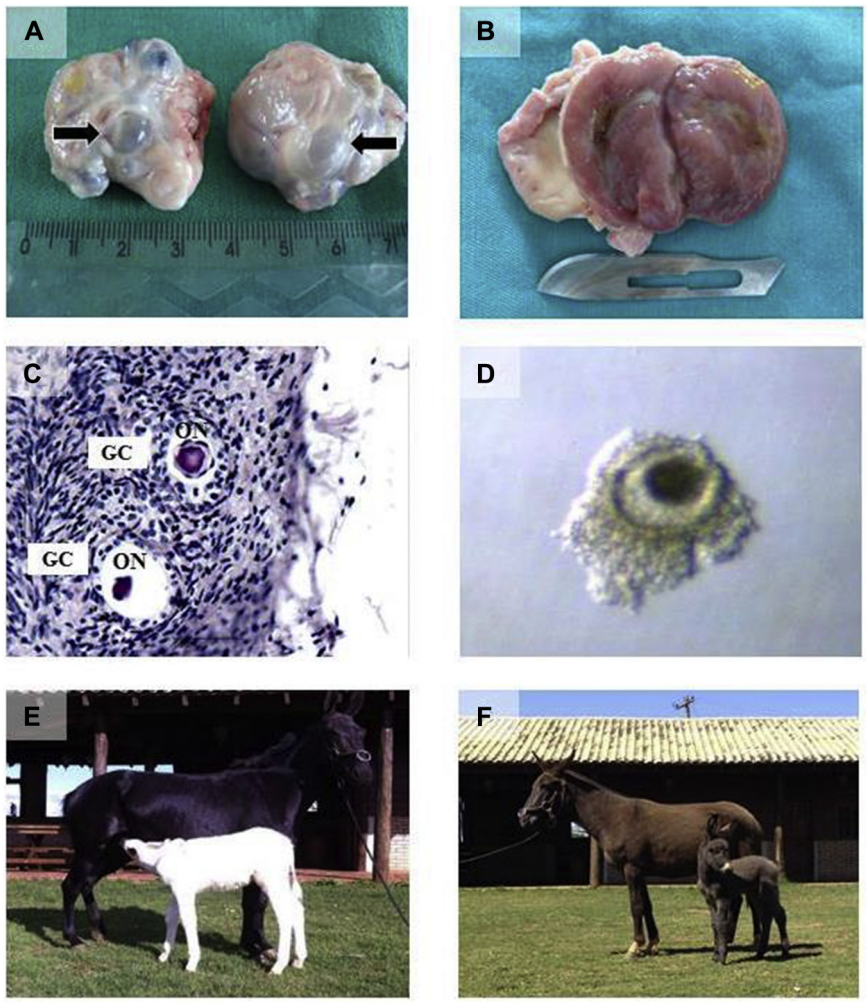

Fig. 1. Reproductive cyclicity in female mules. (A) The presence of follicles in the ovaries of mules (arrows); (B) the presence of corpora lutea in the ovaries of mules; (C) photomicrograph of histologic section of ovarian tissue with primary follicles. Coloration PAS (Periodic Acid Shiff). 40x magnification; (D) oocyte recovered that remained intact with cumulus expansion; (E and F) the female recipients mules with donkey foals.

using the surgical approach [13]. These authors transferred equine embryos and hormonal treatment to induce the estrous cycle and also synchronization of estrus in female mules. After the transfer of nine embryos collected from four mares, these authors reported four pregnancies that resulted in the birth of two foals. In our work, we succeeded in nonsurgical approach and also using spontaneous estrus cycle. The combination of these two aspects will certainly contribute for an easier utilization of mules as embryo recipients.

Embryo transfer between equine and asinine was previously described [14,15]. Donkey embryos were transferred to mares, but the authors reported pregnancy losses, due to few or absent endometrial cups at the placenta. This situation has been reported due to the equine fetal genotype influencing the intensity and the success of the maternal endometrial leukocyte response against the cups [16]. These authors considered that the gonadotropin acts as a protective barrier around the cells of the endometrial cups, avoiding the attack sensitized maternal lymphocytes. In our report, the mules provided healthy foals after eutocic birth, suggesting a normal development of the placenta. In this way, mules with regular estrous cycles emerge as a new alternative as recipient to donkey embryos, probably better than mares.

We also confirmed the excellent maternal ability of mules, as previously described [13]. Right after the birth of the foals, both mules presented strong protective behavior and large amount of milk in the well-developed udders.

5. Conclusions

The presence of corpora lutea, follicles, and oocytes in the ovaries of female mules is associated with characteristics of cyclicity, which is in agreement with the birth of two donkeys after the successful transfer of embryo into mule recipients. Thus, we suggest the mules may be evaluated to be potentially used in reproduction biotechnologies.

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