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Embryo recovery results in Hispano-Arabe horse and Spanish donkey breeds

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ABSTRACT

This study was carried out as part of an embryo preservation program undertaken among Hispano-Arabe (H-a) mares and Spanish (Andalusian and Zamorano-Leones) jennies, both considered to be endangered breeds. Over the course of six years, 61 donor mares and 17 donor jennies were monitored and inseminated with chilled semen from 17H-a stallions and six jackasses. A total of 199 uterine flushings (140 in mares and 59 in jennies) were carried out and the embryo recovery rate was recorded and the effect of various factors such as embryo quality and size, flushing effluent quality and individual effects were analyzed. The ovulation rate was significantly lower in donor mares (1.12) than in jennies (1.86) ($p < 0.001$). Embryo recovery per flush was 35.0% and 40.7% in mares and jennies respectively ($p > 0.05$), and it was influenced by photoperiod ($p < 0.05$). Individual effects were also noted for different stallions and jackasses. The embryo size was significantly affected by day of flush ($p < 0.05$) and embryo morphology ($p < 0.001$). While the effect of a range of variables is described in this study, it is important to emphasize that the endangered nature of the breeds concerned makes it harder to obtain acceptable results in an embryo transfer program, because it is difficult to select the best animals (donors, recipients and stallions), and this may play a role in the results obtained. However, it is necessary to continue the research in this field in order to improve the tools needed to preserve the pool of genetic heritage and diversity.

1. Introduction

Equine species are deeply engrained in Spanish culture and society, and numerous local breeds of both horses and donkeys can be found. While horses are used for riding, sport, herding and tourism, the outlook for donkeys is bleaker, because agriculture has declined and the work that they carried out in the past is now done by agricultural machinery. Many of the horse and donkey breeds are classified as endangered, and to maintain the diversity of these animals it is necessary to develop programs to reduce the genetic drift and the extinction of the breeds. Numerous initiatives can be implemented with the purpose of achieving this goal, among them embryo transfer, an "*ex situ*" strategy for the preservation of endangered animals. This technology has been the subject of a number of studies carried out in horses, with variable results. However, relatively few studies on jennies are to be found in the literature, and those that exist exhibit poor results, since knowledge in this field in extremely limited. The embryo bank is another "*ex situ*" strategy for the preservation of animals (Andrabi and Maxwell, 2007), and cryopreservation methods in equine species have been developed successfully in recent years. Cryopreservation methods, both slow freezing and vitrification, show more satisfactory and promising results when small embryos are used (Vajta and Kuwayama, 2006; Stout, 2012). To obtain the best results, it is necessary to know which factors impinge on embryo recovery, and then improve knowledge about the differences between horses and donkeys.

The Hispano-Arabe horse breed and Spanish (Andalusian and Zamorano-Leones) donkey breed were included in a cryopreservation program because of their relatively small population figures. The Hispano-Arabe horse is a breed that is indigenous to Spain, with 8567 animals registered in the studbook, and is classified as an endangered breed by the Spanish Government (MAGRAMA, 2016). As far as Spanish donkey breeds are concerned, all are in a critical situation, with reduced populations, and are also classified as endangered. A concerted effort is therefore required if the genetic heritage and diversity of these breeds is to be preserved.

The aim of this study was to elucidate which factors impinge on the recovery of embryos, in order to create a germplasm bank for the Hispano-Arabe horse and Spanish donkey breeds.

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2. Materials and methods

2.1. Animals

A total of 61 Hispano-Arabe (H-a) breed mares and 17 Spanish (Andalusian and Zamorano-Leones) breed jennies, between 3 and 19 years old, were selected for an embryo preservation program, and were included in this study. The animals were reared at Las Turquillas farm, located in southern Spain (Ecija, Seville, 37°32´N, 5 °5´W), with a Mediterranean climate. The mares were kept overnight in individual boxes and in paddocks during the day. They were fed with alfalfa and grass hay, supplemented with concentrate, and with free access to water and to trace mineralized salt.

In accordance with the seasonality prevailing in Spain for equine animals, breeding season in mares includes spring transition and ovulatory season, which ran between March and October, coincident with increasing photoperiod. In contrast, during the decreasing photoperiod extended from November to February, mares show fall transition and deep winter anetrus, and reproductive activity is reduced or hindered. Even though jennies do not exhibit seasonal reproductive pattern, increasing and decreasing photoperiods were evaluated.

A total of 17H-a stallions (from 2009 to 2013) and 6 Spanish (Andalusian and Zamorano-Leones) jackasses (from 2014 to 2016) were used. Sperm was collected twice a week by artificial vagina in all males, and motility was contrasted (by sperm analyzer system, ISAS) in order to guaranty the best quality of doses. Only normospermic ejaculates were used. Chilled semen diluted with INRA 96® (IMV Technologies, L'Aigle, France) was used the same day of its collection, with a total of 600×10^6 progressively motile sperm/doses.

2.2. Monitoring and insemination of donors

When signs were detected of donors being in heat, they were checked sonographically every day using a linear-array scanner with a 5.0 MHz probe (Falco, Esaote, Barcelona, Spain) for monitoring uterine status (to discard endometritis) and ovarian activity. They were inseminated when ultrasonographic preeminent features for ovulation were observed in the preovulatory follicle (i.e. diameter around 30–35 mm, triangular shape) and/or uterus (i.e. edema disappearance). After insemination, donors were monitored 24 h later to determine if ovulation had occurred; if not, insemination was repeated 48 h later. The time of ovulation was calculated as occurring half-way between a preovulatory follicle being last observed and the first observation of its absence. In the case of multiple ovulation, the day of the first registered ovulation was used to calculate the embryos age (Blanchard et al., 1999).

2.3. Embryo recovery

A total of 199 uterine flushings (140 in mares and 59 in jennies) were carried out during the study. In order to recover embryos, donors were flushed between day 6.5 and 8.5 (± 0.5 days old at recovery) after ovulation. Only animals difficult to handle were previously sedated with 20 μg/kg detomidine hydrochloride (Domosedan®, Pfizer, Spain) (Huhtinen et al., 1996). Sonographic examination was used to evaluate the ovary (the presence of corpus luteum) and uterine status. The vulva and perineum were cleaned with povidone-iodine scrub, rinsed three times with warm water and dried. Ringer-lactate medium (Braun, Barcelona, Spain) was warmed to 37.5 °C and then it was perfused into the uterus through a Foley-type balloon-tipped catheter (Bioniche Animal Health, Pullman, USA) with a "Y" junction that connects one end to the flush medium and the other end to a filter. The catheter was lubricated with sterile, non-spermicidal vaseline, its tip was placed through the cervix just to the end of cervix, and the balloon was inflated with air. The uterus was flushed four times with 1 l of medium and the recovered fluid was filtered through an in-line filter (Miniflush®, Minitube Iberica, Tarragona, Spain). After flushing, the filter was taken to the lab and the search for embryos was carried out using stereoscopic microscopy (Nikon SMZ645, Japan). Embryos were measured using an eye-piece rule, and quality was determined according to Slade et al. (1985). The embryos were then washed 10 times in a holding medium (Syngro, Bioniche Animal Health, Pullman, USA) for their subsequent vitrification.

Efflux clarity was recorded in all mares after uterine flushing, and graded as clear (type 1), and either partially (type 2) or highly (type 3) cellular and/or cloudy, depending on the concentration of cells, mucus, debris or other matter contained in the efflux.

After embryo recovery, PGF2α (2 ml IM, Prosolvin, Virbac, Spain) was administered to donors in order to induce luteolysis.

2.4. Statistical analysis

The determination of sample size was based on the number of females recorded in each Stud Book in the last year (aprox. 3092 in H-á mares and 1007 in Spanish jennies) (MAGRAMA, 2016) and on the basis of a cumulative rate of 60%. It was established a power of 80%, with a significance level set at $\alpha = 0.05$, and an error of 7%. Seventy-nine animals per group would be required. Then, assuming that horse and donkey embryo recovery is quite similar, and due to the difficulty to attain this sample size, the observations were completed with embryo flushings carried out in both species. Due to the mentioned difficulties, a pilot study was planned.

A descriptive statistic was used to determine the values corresponding to ovulation rate, embryo recovery rate, embryo quality and size, flushing effluent quality and percentage of males and females with productive flushing.

The effects on the ovulation rate of species (horse or donkey), age of females (≤ 5; 6–9; ≥10 years), and photoperiod (positive photoperiod: March-October; negative photoperiod: November-February) were all analyzed. The effects on embryo recovery per flushing and per ovulation of the species, female age, photoperiod, flushing quality (good, medium, poor), number of ovulations (1, 2, 3 or 4) and days after ovulation (6.5, 7.5 and 8.5) were also evaluated. The one-sided chi square test was carried out.

The effects on embryo quality of species, female age, number of ovulations, photoperiod, days after ovulation and flushing quality were analyzed. The influence on embryo diameter of species, female age, number of ovulations, photoperiod, days after ovulation and embryo morphology (morula, blastocyst and expanded blastocyst) was also investigated. Levene's test was used to evaluate data normality. Since significance was observed, it was assumed that variances were different, in which case data distribution was not normal, and a non-parametric test was used. The Kruskal-Wallis H test was carried out, and a post-hoc LSD test was used when significant differences were detected.

The SPSS 11.0 package (SPSS, Chicago, IL, USA) was used for statistical analysis. All results are presented as mean ± SD. The significance level was set at $p \leq 0.05$.

3. Results

The average ovulation rate per female was significantly lower in mares (1.12 ± 0.33) than in jennies (1.86 ± 0.71) ($p < 0.001$). While mares showed few multiple (double) ovulations (17/140; 12.1%), they were much more commonly found in jennies (32/46; 69.5%), where they consisted of double, triple or quadruple (54.2, 13.6 or 1.7%, respectively) ovulations.

However, the ovulation rate per female during different photoperiods and in donor age groups did not show significant differences in either the mare or jenny groups ($p > 0.05$).

The average percentage of embryo recovery per flushing in the present study was 35.0% and 40.7% in mares and jennies respectively.