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Effect of month of conception on fertility of mares inseminated with jackass semen

[*Influência do mês da concepção sobre a eficiência reprodutiva de éguas inseminadas com sêmen asinino*]

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ABSTRACT

Fertility obtained by cross-breeding mares (*Equus caballus*) with jackasses (*Equus asinus*) was evaluated. Two extenders, containing skim milk-glucose or egg yolk-glycine were used to study the fertility of mares inseminated with diluted jackass semen (T1 and T2) or diluted and cooled semen at 5°C for 12 hours (T3 and T4). A total of 272 cycles of 208 mares of undefined breeds were evaluated, being uniformly distributed between groups. The cycles were controlled by transrectal palpation and teasing, and mares were inseminated every Tuesday, Thursday and Saturday (three times/week), from the detection of a follicle with 3.0 to 3.5cm diameter in one of the ovaries until ovulation. Pregnancy was detected using transrectal palpation, teasing and ultrasound exams every 14 days. The extenders had no effect on fertility (P>0.05). Pregnancy rates for the first cycle were 64.52%, 61.11%, 50.72% and 54.17% and pregnancy rates/cycle were 63.64%, 54.55%, 52.69% and 47.06%, respectively, for T1, T2, T3 and T4. Differences in pregnancy loss rates between groups and effect of month of conception on fertility were found. Pregnancy loss rates were significantly higher (P<0.05) in January (38.46%) and in February and March (52.38%), with an average of 33.09%. The results indicate that mares conceiving at the end of the physiological reproduction time, carrying a mule embryo, are more susceptible to pregnancy loss.

Keywords: fertility, pregnancy loss, intra and interspecific cross-breeding, cooled semen

RESUMO

Avaliou-se a eficiência reprodutiva de cruzamentos interespécies entre fêmeas equinas e reprodutores asininos da raça Pêga. Para tal, estudou-se o efeito de dois diluidores, à base de leite em pó desnatado-glicose ou glicina-gema de ovo, sobre a fertilidade de éguas inseminadas com sêmen fresco diluído (T1 e T2) ou diluído e resfriado a 5°C por 12 horas (T3 e T4). Foram utilizados 272 ciclos de 208 éguas mestiças, distribuídas uniformemente entre os tratamentos, após agrupamento por idade e categoria reprodutiva. Os ciclos foram acompanhados por palpação retal e rufiação, sendo as inseminações realizadas às terças, quintas e aos sábados (três vezes por semana), a partir da detecção de um folículo de 3,0 a 3,5cm de diâmetro, em um dos ovários, até a ovulação. As gestações foram acompanhadas por palpação transretal, rufiação e por ultrassonografia, realizada a cada 14 dias. As taxas de gestação obtidas, ao primeiro ciclo, não diferiram (P>0,05) entre os tratamentos, sendo de 64,52% para T1, 61,11% para T2, 50,72% para T3 e 54,17% para T4. Da mesma maneira, as taxas de gestação por ciclo também não foram influenciadas (P>0,05) pelos tratamentos, com valores de 63,64%, 54,55%, 52,69% e 47,06% para T1, T2, T3, e T4, respectivamente. No que diz respeito às taxas de perdas gestacionais, obtidas por meio das taxas de perdas e de perdas ajustadas, houve influência (P<0,05) dos tratamentos.

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Observou-se, ainda, efeito do mês da concepção, com taxas de perda superiores para os meses de janeiro (38,46%) e de fevereiro e março (52,38%), sendo a média de 33,09%. Sugere-se que a redução dos padrões de secreção do LH, ao final da estação de monta, responda pelas maiores taxas de perdas gestacionais do cruzamento interespécie, notadamente naquelas éguas que conceberam no final da estação de monta fisiológica dos equinos.

Palavras-chave: eficiência reprodutiva, perdas gestacionais, cruzamentos intra e interespécies, sêmen resfriado

INTRODUCTION

Mule production success depends on the cross-breeding between jackasses and mares with high genetic potential, coupled with factors such as age and reproductive capacity (Jordão *et al*., 1954). The feasibility of modern horse husbandry requires technological advances that increase productivity, making it more profitable. In this context, embryonic and fetal death are major factors in subfertility and reduced reproductive efficiency in mares, causing significant economic losses to horse owners (Ball, 1993). Despite considerable advances in equine reproduction during the last 20 years, embryonic death and fetal losses remain poorly understood, particularly with regard to interspecific mule pregnancies.

The literature is sparse regarding the reproductive efficiency of mares carrying a mule embryo. Thereupon, Jordão *et al*. (1954) had a foaling rate of only 35.9% in mares kept under pasture in natural breeding with jackasses for 14 consecutive breeding seasons. After this study, only in 2005, Boeta and Zarco described a pregnancy loss rate of 36.8% in mares with interspecies pregnancies (jackass x mare) which was significantly higher than intraspecies pregnancies (horse x mare) with a loss rate of 21.4%. A possible cause for this lower fertility is the low equine chorionic gonadotropin (eCG) production by mares carrying hybrid embryos (Pigoniowa, 1962), due to inadequate development of the endometrial cups, associated with their premature regression (Allen *et al*., 1987).

In response to the eCG secretion, the primary *corpus luteum* (PCL) of pregnant mares increases in size (Squires *et al*., 1974), raises the progesterone and starts the estradiol production (Daels *et al*., 1991). In the ovaries, besides the primary *corpus luteum* luteotropic stimulation, the eCG induces the secondary *corpus luteum* (SCL) formation (Squires *et al*., 1974). This is determined by the biological effect of eCG binding to LH receptors. Thus, eCG is responsible for the increase in plasma progesterone concentrations observed after 35-40 days of gestation (Allen, 1984; Allen *et al*., 1987).

According to Allen (2001), after the 40th day of gestation, the eCG protects pregnant mares from PGF2α luteolytic effect. Thus, the low production and secretion of eCG in mares with interspecies pregnancies associated with lower production of progesterone makes these females more susceptible to pregnancy loss.

In this context, the aims of this study were to evaluate the reproductive efficiency of mares inseminated with diluted or diluted and cooled jackass semen, through the fertility and pregnancy loss analysis and evaluate the breeding season month effect on pregnancy loss incidence in mares impregnated with jackass semen.

MATERIALS AND METHODS

The experiment was conducted at the Váu Farm in the city of Lagoa Dourada, state of Minas Gerais, Brazil, from October 2007 to March 2008.

The results of 272 cycles of 208 mares (2.5 to 18 years old) from different reproductive status (maiden, barren, lactation or in "foal heat") were used. The mares were distributed randomly, after grouping by age and reproductive status. Seventy-seven cycles of 67 mares were inseminated with fresh diluted semen in nonfat dry skim milk-glucose (T1) or egg yolk-glycine (T2) extenders. For insemination with diluted and cooled semen, in the same extenders mentioned earlier, 195 cycles of 141 mares were assigned to two treatments (T3 and T4, respectively).

Five Pêga jackasses (*Equus asinus*) were used as semen donors. In treatments 1 and 2 four Pêga jackasses represented by J1, J3, J4, J5, (15, 15, 7 and 9 years old), respectively, were used. For treatments 3 and 4, five Pêga jackasses were used, including ones from the previous treatments plus an 8-year-old jackass, represented by J2. The treatments were:

T1 - Mares inseminated with fresh semen diluted in skim milk-glucose (Kenney *et al*., 1983) extender, using an insemination dose of 20mL and 400 x 10^6 motile spermatozoa (n=33);

T2 - Mares inseminated with fresh semen diluted in egg yolk-glycine (Foote, 2002) extender, using an insemination dose of 20mL and 400 x 10^6 motile spermatozoa (n=44);

T3 - Mares inseminated with semen diluted in skim milk-glucose (Kenney *et al*., 1983), using an insemination dose of 20mL and 400 x 10^6 motile spermatozoa, previously cooled and stored for 12 hours at 5° C in a special container (Palhares, 1997) (n=93);

T4 - Mares inseminated with semen diluted in egg yolk-glycine (Foote, 2002) extender, using an insemination dose of 20mL and 400 x 10^6 motile spermatozoa, previously cooled and stored for 12 hours at 5°C in a special container (Palhares, 1997) (n=102).

Transrectal palpation was done every three days until a follicle with a 2.0-2.5cm diameter was detected in one of the ovaries, being thereafter conducted daily. Once a dominant follicle with a 3.0-3.5cm diameter was detected, artificial inseminations were performed on fixed days (Tuesdays, Thursdays and Saturdays) until ovulation. A dose of 1.667 UI/mare of Human Chorionic Gonadotropin (hCG) was used to induce ovulation.

Daily reproductive exams stopped after ovulation until pregnancy diagnosis by ultrasonography and teasing, the latter being conducted from the 15th day after ovulation. Ultrasonography examinations, using a high resolution scanner equipped with a 7.5 MHz linear array transducer (SSD500, Aloka) were initiated in a minimum of 11 to a maximum of 16 days post-ovulation. Each positive pregnancy was confirmed at least twice. Mares that lost pregnancies with less than 35 days returned to the breeding program. All mares were evaluated until the end of the breeding season and the foaling data was collected, with the exception of six animals.

The mares were maintained on pasture for the entire experimental period, and had free access to hay, with molasses grass being predominant (*Melinis minutiflora*), water and trace mineralized salt. The Jackasses were fed with 20kg of chopped elephant grass (*Pennisetum purpureum*), distributed twice a day, 4kg of dry feed ration produced on the farm and had free access to water and trace mineralized salt.

The variance analysis was used for quantitative variables (number of cycles and number of ovulations/month) and the Student t test was used for the comparison of two means. When comparing more than two means, the Student-Newman-Keuls (SNK) test was used.

The proportional data (pregnancy rate/cycle, total pregnancy rate, loss rate, adjusted loss rate, foaling rate, adjusted foaling rate, pregnancy rate/cycle/month) were tested using the chi-square test to detect differences between treatments.

RESULTS AND DISCUSSION

Table 1 shows the results obtained in treatments 1, 2, 3, and 4. Pregnancy rate on the first cycle, and pregnancy rate/cycle did not differ ($P>0.05$) between treatments. Total pregnancy loss rates obtained through loss and adjusted loss rates were influenced ($P<0.05$) by the treatments, similar to what was found for foaling and adjusted foaling rates.

Comparing the present results for T2 and T4 with those found in the literature, which also used extenders based on egg yolk, there was similarity in pregnancy rates as shown by Silva (1988), who obtained 52.4%, 52.2% and 68.5% of pregnancy rates, for the first cycle, studying 240 mares inseminated with diluted jackass semen in three consecutive breeding seasons. Palhares *et al*. (1986) reached 57% of pregnancy rate, for the first cycle, using jackass semen to inseminate jenny asses. However, Ferreira (1993) obtained better results, with 82.7% of pregnancy rate/cycle, using cooled jackass semen, diluted in egg yolk-lactose extender. However, the author

conducted screening combining the best jackass and extender "*in vitro"*, to perform the inseminations. This could be responsible for the high pregnancy rates found.

Our results using jackass semen, diluted (T2) or diluted and cooled (T4) using egg yolk-glycine extender, were similar (P>0.05) when compared with treatments using skim milk-glucose (T1 and T3).

Considering that the egg yolk-glycine extender was used for the first time to dilute equid semen and that there are no other reports in the literature, it is important to emphasize the need for further studies "*in vivo*" to reveal the real impact on mare fertility.

Table 1. Reproductive variables of mares inseminated with diluted or diluted and cooled jackass semen, from conception to birth

Variable	Treatment				Total
	T1	T2	T3	T4	
Number of Mares	31	36	69	72	208
Number of Cycles	33	44	93	102	272
Pregnancy rate for the 1st cycle (%)	64.52 (20/31)	61.11 (22/36)	50.72 (35/69)	54.17 (39/72)	55.77 (116/208)
Pregnancy rate/cycle (%)	63.64 (21/33)	54.55 (24/44)	52.69 (49/93)	47.06 (48/102)	52.21 (142/272)
Pregnancy loss rate (%)	61.90 (13/21)^{a}	41.67 (10/24)^{ab}	14.29 (7/49)^{c}	31.25 (15/48)^{bc}	31.69 (45/142)
Pregnancy adjusted loss rate (%)	61.90 (13/21)^{a}	45.45 (10/22)^{ab}	15.22 (7/46)^{c}	31.91 (15/47)^{bc}	33.09 (45/136)
Foaling rate (%)	38.10 (8/21)^{c}	50.00 (12/24)^{bc}	79.59 (39/49)^{a}	66.67 (32/48)^{ab}	64.08 (91/142)
Adjusted foaling rate (%)	38.10 (8/21)^{c}	54.55 (12/22)^{bc}	84.78 (39/46)^{a}	68.09 (32/47)^{ab}	66.91 (91/136)
Unregistered births	0	02	03	01	06

Means followed by different letters on the line differ statistically (P<0.05).

Table 1 shows similar (P>0.05) pregnancy rates/cycle for all treatments (T1, T2, T3 and T4). The use of nonfat dry skim milk as a component of the extender for semen was used by Kenney *et al*. (1983), yielding the pregnancy rate of 58% from mares inseminated with diluted stallion semen. Leite (1994) using jackass fresh semen, diluted in nonfat dry skim milk-glucose, reached 76.92% and 64.70% (P>0.05) pregnancy rates/cycle for the 0-24 and 24-48 hour interval from insemination to ovulation, respectively. Results were similar to those found in the present study for T1.

Pregnancy rates obtained in this experiment, for both fresh diluted or diluted and cooled jackass semen were superior to those reported by Jordão *et al*. (1954), who found just 35.9% of foaling rate, for mares served in natural breeding by jackasses. Conditions associated with reproductive and nutritional management, such as daily follicular control and artificial insemination can explain the good results obtained in the present work.

Pregnancy rates/cycle were similar (P>0.05) for November, December, January and February/March (Table 2).

Regarding pregnancy loss incidence, a month effect (P<0.05) was observed (Table 2). The highest pregnancy loss incidence was recorded in mares pregnant in February/March months, that correspond to the end of the breeding season in the southern hemisphere, compared with those conceived in November and December, the beginning of the breeding season. Mares who conceived in January had similar pregnancy loss rates (P>0.05) as those observed in mares that conceived in November, December and February/March (Figure 1).

Table 2. Month influence on ovulation, pregnancy rate/cycle and pregnancy loss rate (assessed from conception to birth) of mares inseminated with diluted or diluted and cooled jackass semen

Variable	Month				
	November	December	January	February/March	Total
Pregnancy rate/cycle (%)	42.86 (30/70)	55.41 (41/74)	51.92 (27/52)	57.89 (44/76)	52.21 (142/272)
Unregistered births	-	03	01	02	06
Pregnancy loss rate (%)	20.00 (6/30)[b]	18.42 (7/38)[b]	38.46 (10/26)[ab]	52.38 (22/42)[a]	33.09 (45/136)

Means followed by different letters on the line differ statistically (P<0.05).

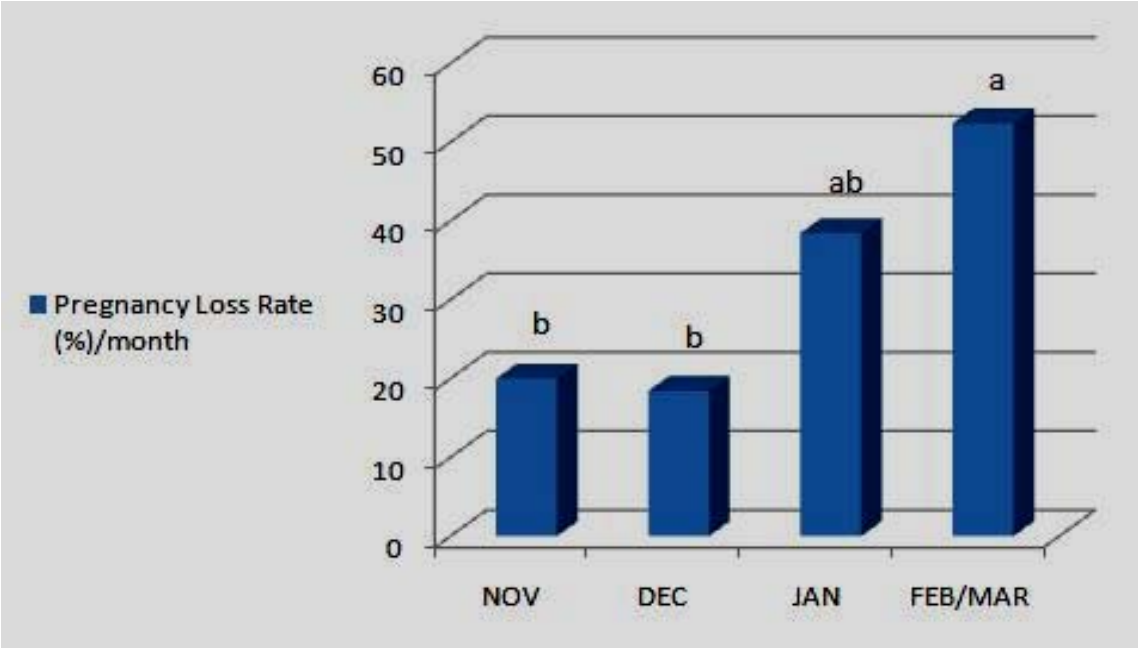

Figure 1. Pregnancy loss rate of mares inseminated with diluted or diluted and cooled jackass semen according to the month ovulation occurrence, assessed from conception to birth. Means followed by different letters differ statistically (P<0.05).

In this experiment a high percentage (33.09%) of pregnancy loss was observed (Table 2). However, it was not possible to determine the exact gestation period. It should be pointed out that a higher number of mares was used in the present experiment to characterize the losses compared to other similar studies. Boeta and Zarco (2005) used a total of 47 pregnant mares, being 28 horse embryos and only 19 mule embryos, compared to the 208 mares carrying a mule embryo, evaluated in this experiment.

High loss rates were also observed by Jordão *et al*. (1954), who obtained only 35.9% birth rate. Boeta and Zarco (2005) evaluated mares until 150 days of pregnancy resulting from inseminations with jackass semen and found a high pregnancy loss incidence (36.8%), being all losses observed after 40 days of ovulation and thus, classified as fetal losses. They found higher pregnancy losses in mares carrying hybrid mule than for mares impregnated by a stallion (21.4% of pregnancy loss). In another study, Ferreira (1993) observed 5.5% pregnancy losses between 30 and 50 days of pregnancy from mares inseminated with cooled jackass semen, similar to other rates described for mares with intra-species pregnancies. Similarly, Garg and Manchanda (1986) observed a 10.4% abortion rate in mares covered by jackasses, a higher incidence than observed for mating involving stallions, which was 7.8%. Boeta and Zarco

(2010) demonstrated a higher abortion incidence in mares inseminated with jackass semen (30%) versus only 5% in mares inseminated with stallion semen, until 120 days of pregnancy. It should be noted that the authors registered different gestational periods, which may have caused the result variability. There are no reports in the literature about full pregnancy monitoring, which makes it difficult to determine the exact period in which mares with interspecies pregnancies (mare x jackass) are more likely to lose pregnancy.

In general, the studies show higher loss values in hybrid pregnancies than those obtained from mares impregnated by stallions. Using stallion semen, Ginther (1992), Papa *et al.* (1998) and Vanderwal (2011) found a pregnancy loss rate between 5-16% in equine intraspecies pregnancies. Striking pregnancy losses like in the study of Boeta and Zarco (2005, 2010), Jordão *et al*. (1954) and in the present study, were also observed by Lucas *et al.* (1991), studying feral horses. The authors observed an average rate of 32.3% of pregnancy loss after 120 days of gestation. The highest loss rates (70% and 46.3%) were recorded for the group of fillies that were one and two years old, respectively. The authors attributed these findings to the immaturity of pubescent females, since mares with three and four years of age had pregnancy loss rates of 16.7% and 5.6%, respectively, being similar to those observed for mares served by stallions (Ginther, 1992).

Hybrid gestations between different species of Equidae result in significant changes in the formation, maintenance and regression of the endometrial cups. Mares pregnant with mule embryos exhibit serum eCG six to ten times lower than mares carrying horse embryos. Moreover, the eCG secretion period is shorter and becomes undetectable in the bloodstream before 80 days of gestation (Boeta and Zarco, 2005; Boeta, 2008). These authors reported lower ($P<0.05$) eCG circulating in mares inseminated with jackass semen, when compared to mares inseminated with semen from stallions. In addition, it was observed that the concentration of this hormone returned to baseline levels earlier in mares carrying mule embryos. It was shown that circulating progesterone concentrations did not increase between days 35 and 49 of pregnancy, as noted in mares carrying horse embryos, indicating that low eCG concentrations do not stimulate luteal function in interspecies pregnancies. However, Boeta (2008) confirmed that even with low eCG concentrations, the supplementary *corpora lutea* was formed in mares inseminated with jackass semen, despite the later and smaller number occurrence.

According to Boeta and Zarco (2005), the low eCG and progesterone concentrations in mares carrying mule embryos did not affect embryo survival, since there were no differences in the concentrations of these hormones between mares that had normal pregnancies and births and those that lost. This finding is in agreement with Allen (1984, 2001) who concluded that eCG is not essential for the maintenance of pregnancy in mares.

Even with low progesterone concentrations, the growth rate of the embryonic vesicle of mares inseminated with jackass semen was similar to that observed in mares inseminated with stallion semen (Ginther, 1986). This indicates that minimal concentrations of progesterone may be sufficient to maintain pregnancy in the mare (Allen, 1984). Thus, the low progesterone levels observed in mares carrying mule embryos, in most cases, is sufficient to reach the minimal for the maintenance of pregnancy, as well as for normal embryonic development (Boeta and Zarco, 2005).

In interspecies pregnancies, premature regression of the endometrial cups does not induce abortion. However, the humoral and cellular immune response against antigens of the chorionic girdle (Allen, 2001) can cause damage to the placenta, affecting the survival of the conceptus. Pregnancy loss in mares carrying mule embryos was associated with failure in the formation of microcotyledons of the diffuse placenta, as well as with the intense maternal leukocyte response in the endometrial area in direct contact with the fetal membranes (Allen, 2001). Thus, the occurrence of failures in placental development of these pregnancies cannot be ruled out (Boeta and Zarco, 2005).

Allen (2001) proposed that after the 40th day of pregnancy, the eCG protects the mare against PGF-2α luteolytic effects. To confirm this hypothesis, Cantón (2008) administered a

luteolytic drug to mares carrying a mule or a horse embryo at 90 days of pregnancy. Serum progesterone and eCG were measured before and after administration of the luteolytic drug. Results showed a positive correlation ($P<0.05$) between eCG concentrations before administration, and the progesterone concentrations 48 hours after treatment. Abortion was observed only in a mare carrying a mule embryo, concluding that the mares with hybrid pregnancies are more likely to have PGF-2α luteolytic effect.

Arias (2011) also demonstrated that mares carrying a mule embryo are more susceptible to pregnancy loss. The author administered a GnRH antagonist to mares gestating horse or mule embryos and there was no abortion occurrence between mares with horse pregnancies. However, the pregnancy loss rate reached 62.5% (5/8) in mares with mule pregnancies.

Allen (1975) observed the influence of reproductive seasonality of mares on ovarian activity, characterized by ovarian size, number of follicles and ovulation rate during pregnancy. The mares that conceived between April and July showed high ovarian activity at 40 days of pregnancy, similar to the higher levels of circulating eCG. Ovarian activity was maintained after the increase of the eCG concentrations. On the other hand, in mares that conceived between July and February, eCG increases were not accompanied by an increase in ovarian activity, as observed in other mares. Based on these observations, the author considered that the eCG act synergistically with pituitary gonadotropins, that are responsible for the primary stimulation on ovarian activity in pregnant mares. In 1984, Allen suggested that pituitary LH can maintain the luteal function in the absence of eCG in mares carrying mule embryos.

Trying to assess the role of eCG in pregnant mares inseminated with stallions or jackasses, Boeta and Zarco (2012) compared the primary and supplementary *corpora lutea*, in both groups, as well as the concentrations of eCG and progesterone. No differences were found in progesterone or eCG concentrations between mares with mule pregnancies that accumulated different numbers of supplementary *corpora lutea*. The same was not observed in the group of mares with horse pregnancies, since it was found that progesterone and eCG concentrations were proportional to the number of supplementary *corpora lutea* formed. The authors attributed the fact to a possible action of pituitary LH in the luteal activity in mares with mule pregnancies, producing similar progesterone concentrations in all cases, regardless of the number of supplementary *corpora lutea* present in the ovaries.

Boeta and Zarco (2005) postulated that mares that conceive early in the breeding season pass through the first trimester of pregnancy with no suppression of LH. Thus, the hormone concentration would be enough to provide luteotropic stimulus able to maintain progesterone concentrations above the minimum threshold necessary for the maintenance of pregnancy in mares carrying hybrid embryos. This proposal finds support in a study conducted previously by Snyder *et al*. (1979) who detected lower concentration of LH in the last ovulation of the breeding season, compared to the previous one, emphasizing the seasonal effect on LH concentrations. Thus, eCG may be part of a mechanism developed by the mares to replace the luteotropic effect of pituitary LH when conception occurs at the end of the ovulatory season.

Moberg (1975) proposed that the pregnancy loss increased with the use of techniques involving semen manipulation in relation to natural mating. The higher incidence of pregnancy loss (61.90%) in this experiment (Table 1), however, was associated with insemination with fresh diluted semen in skim milk-glucose (T1), and the lowest incidence (15.22%) associated with use of the same extender, but with cooled semen (T3). It is suggested that the higher incidence of pregnancy loss, observed in mares inseminated with fresh diluted semen, is due to the conception month effect, since inseminations with fresh diluted semen (T1 and T2) were conducted from January to March 2008, and inseminations with cooled semen (T3 and T4) were conducted at the beginning of the breeding season, from October 2007 to January 2008.

Thus, it is understood that mares carrying hybrid embryos (mules) show disabled patterns of eCG and are more susceptible to the occurrence of pregnancy loss when served/inseminated at the end of the breeding season. Studies should be

done to reduce these losses, either by exogenous administration of hormones to help the formation of supplementary *corpora lutea*, or by other mechanisms that maintain adequate concentrations of progesterone to support the pregnancy. Mares should be evaluated at more frequent intervals, in more complete studies, including data with measurements of eCG, LH and progesterone, as well as the evaluation of the quantitative and qualitative characteristics of the endometrial cups of mares with mule pregnancies.

In conclusion, this study has shown that extenders based on skim milk or egg yolk are good choices for jackass semen, with satisfactory fertility results. It was also concluded that a higher frequency of pregnancy loss was associated with conception in the final months of the breading season.

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