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CLINIC AND SURGERY

Intrauterine infusion of platelet-rich plasma increases embryonic recovery rates in older donor mares

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ABSTRACT: Platelet-rich plasma (PRP) has anti-inflammatory and regenerative properties with the potential use in advanced age mares uterine alterations. Our objective was to evaluate the effect of intrauterine PRP infusion in older embryo donor mares on embryo recovery rate. Endometrial biopsy was performed for histopathological classification, once, in 18 mares aged 18~25 years. Mares were examined routinely for estrus monitoring. During each estrus phase, uterine cytology was collected and evaluated. When a 35 mm follicle and edema ≥ 2 was detected, ovulation was induced using 1 mg deslorelin acetate, followed by intrauterine infusion, according to treatment: 1) PRP: intrauterine infusion of 5 mL of PRP, and 2) Control: intrauterine infusion of 5 mL of Lactated Ringer's solution. Within 24 h of ovulation induction and intrauterine infusion, mares were inseminated with fresh semen and subsequently 24 h ovulation was detected. Eight days after ovulation, embryos were recovered and classified. Statistical analyses were performed using GraphPad Prism 9® software. In histological classification, IIA mares presented higher embryonic recovery rate in the PRP group than in Control (P = 0.04). In cytological evaluation, no significant difference was observed between groups. A total of 49 embryo recovery attempts were performed, in Control group recovery rate was 21.7% (5 embryos from 23 flushes) while in PRP was 34.6% (9 embryos from 26 flushes), with significant difference in PRP infusion number of recovered embryos (P = 0.04). Intrauterine infusion of autologous PRP, at the time of ovulation induction, increases the chance of embryonic recovery in older embryo donor mares.
Key words: autologous PRP, embryo transfer, endometrium, inflammatory response, post-breeding endometritis.

Infusão intrauterina de plasma rico em plaquetas aumenta as taxas de recuperação embrionária em éguas doadoras de embrião idosas

RESUMO: O plasma rico em plaquetas (PRP) possui propriedades anti-inflamatórias e regenerativas, sendo uma potencial alternativa para éguas idosas com alterações uterinas. Objetivamos avaliar o efeito da infusão intrauterina de PRP na taxa de recuperação embrionária em éguas doadoras de embriões idosas. Biópsia endometrial foi realizada uma única vez, para classificação histopatológica, em 18 éguas, com idade entre 18-25 anos. As éguas foram monitoradas para detecção do estro, e durante o estro, a citologia uterina foi coletada. Quando um folículo ≥ 35 mm e edema ≥ 2 foram detectados, a ovulação foi induzida com 1 mg de acetato de deslorelina, seguida pela infusão intrauterina, de acordo com o grupo experimental: 1) PRP: 5 mL de PRP, e 2) Controle: 5 mL de Ringer Lactato. Após indução da ovulação e infusão intrauterina, as éguas foram inseminadas com sêmen fresco. Oito dias após a ovulação, os embriões foram recuperados e classificados. A análise estatística foi realizada no software GraphPad Prism 9®. Na classificação histológica, éguas IIA apresentaram maior taxa de recuperação embrionária no grupo PRP em comparação ao controle (P = 0,04). Na avaliação citológica, não houve diferença significativa entre os grupos. No total, foram realizadas 49 tentativas de recuperação embrionária, com taxa de recuperação de 21,7% no grupo Controle (cinco embriões de 23 lavados) e 34,6% no PRP (nove embriões de 26 lavados), demonstrando diferença significativa na recuperação embrionária no grupo PRP (P = 0,04). A infusão intrauterina de PRP autólogo, no momento da indução da ovulação, aumenta a chance de recuperação embrionária em éguas doadoras com idade avançada.
Palavras-chave: PRP autólogo, transferência de embriões, endométrio, resposta inflamatória, endometrite pós-reprodutiva.

INTRODUCTION

In equine breeding programs, it is estimated that 10-25% of mares are advanced in age, and this relatively high percentage of older females may be associated with the fact that genetically superior and valuable breeders are maintained for a longer period in breeding programs, aiming to obtain a greater number of descendants (BAKER et al., 1993). Mares can reproduce despite advancing age (BURNS, 2016; HOROHOV et al., 2002; TRAUB-DARGATZ, 2006), in contrast to what is commonly observed in human and monkeys females (TE VELDE, 2002; VIDEAN et al., 2008). However, reproductive potential

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decreases with increasing age (CARNEVALE, 2006; SCOGGIN, 2015).

Uterine changes are directly related to age and fertility of mares (RICKETTS & ALONSO, 1991). Endometrial fibrosis is a progressive condition that tends to worsen with age (WOODWARD et al., 2012) and is associated with repeated episodes of uterine inflammation and infection (TROEDSSON, 1997). Healthy mares present a physiological and transient uterine inflammatory response after the introduction of sperm; however, females with endometrial fibrosis, which are susceptible to persistent post-breeding endometritis, present a delayed and prolonged uterine inflammatory response (CARNEVALE, 2006). This inflammation compromises the endometrium, making the uterine environment hostile to the embryo and consequently negatively affecting embryonic recovery and pregnancy rates (TROEDSSON & LIU, 1991; TROEDSSON, 1997).

In recent years, the lack of response to conventional therapies, together with the increased incidence of antibiotic-resistant bacteria, has resulted in the development of alternative treatments for mares susceptible to endometritis, particularly in cases of persistent post-breeding endometritis (SCOGGIN, 2016). In this context, platelet-rich plasma (PRP), a derivative of whole blood plasma with a high platelet content, has been used in humans and animals as an alternative to overcome reproductive problems. This byproduct has anti-inflammatory, regenerative, and antimicrobial properties (ÁLVAREZ et al., 2011; SEGABINAZZI et al., 2021; SOARES et al., 2021) and contains important growth factors in tissue repair, with mitogenic, chemotactic, and neovascular properties (GONSHOR, 2002; KIM et al., 2014).

Based on a current literature review, there is substantial evidence that intrauterine PRP is beneficial in immunomodulating the uterine inflammatory response in mares, especially those susceptible to post-breeding persistent endometritis (PASCH et al., 2021). However, to the best of our knowledge, no specific studies with advanced aged mares and embryo recovery were found. Therefore, the objective of the present study was to evaluate the effect of intrauterine infusion of autologous PRP on the embryo recovery rate in older embryo donor mares.

MATERIALS AND METHODS

Animals

We selected 18 mares, aged between 18~25 years, Quarter Mile mixed-breed, with body condition score 3 (scale 1-5), maintained under extensive system, in a stud farm in Rondon do Pará, Pará, Brazil (Latitude: 4°46'31" South, Longitude: 48°4'3" West), during two consecutive breeding season. The mares were excluded from reproductive programs in the previous season, due to a failure in embryo recovery during the two preceding breeding seasons.

Sequence of procedures performed in old donor mares are illustrated in figure 1.

Endometrial biopsy

At the beginning of breeding season, during the first estrous phase, endometrial biopsy samples were collected from each mare once, followed by immersion in 10% formaldehyde. Histopathological evaluation was performed using hematoxylin and eosin staining to

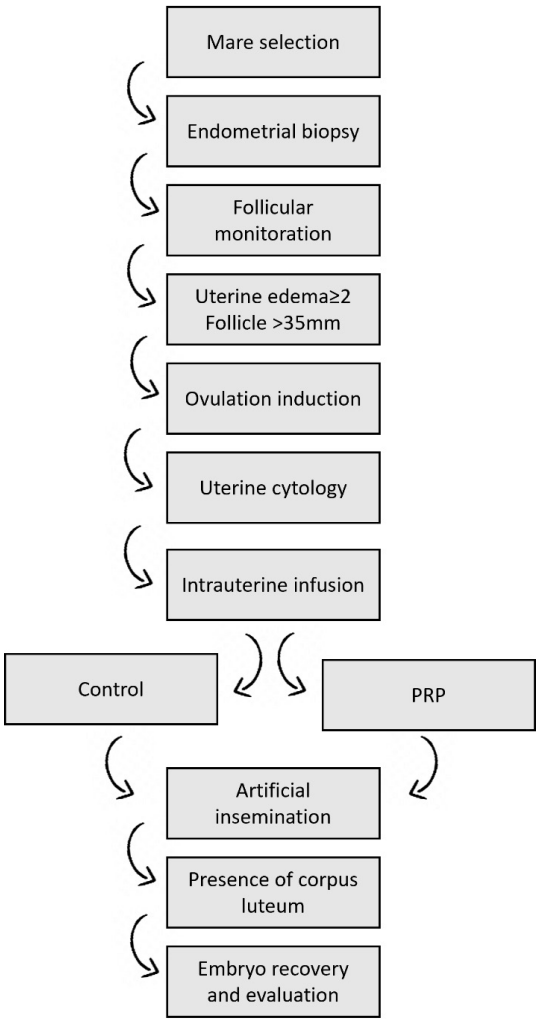

Figure 1 - Flowchart illustrating the sequence of procedures performed in old donor mares.

classify the endometrial quality into grades I, IIA, IIB, and III, as described by SNIDER et al. (2011).

Estrous monitoring and uterine cytology

During breeding season, mares were routinely examined by palpation and transrectal ultrasonography to evaluate the uterus, ovaries, and estrous monitoring.

During each estrous phase, cytological samples were collected with a cytobrush collector, introduced manually through the vulva and cervix, guided into the uterus. Cytobrush was slightly slid by the microscopic slide, performing the smear, stained with rapid panoptic, and cytological evaluation was performed as described by COUTO & HUGHES (1984), classified as negative, mild, moderate and severe.

Ovulation induction

When a 35 mm follicle and endometrial edema ≥ grade 2 were detected, ovulation induction was performed with 1 mg of deslorelin acetate (Sincrorelin, Ouro Fino®) intramuscular, followed immediately by intrauterine infusion, according to the PRP and Control group.

Intrauterine infusion - PRP and Control

To prepare autologous PRP, blood was collected via jugular venipuncture and transferred to tubes containing 3.2% sodium citrate. The sample was prepared as described by SEGABINAZZI et al. (2021), where 50 mL of blood was divided into 15 mL Falcon tubes and centrifuged at 400 × g for 15 min. The resulting plasma was transferred to 15 mL Falcon tubes and centrifuged at 1000 × g for 10 min. After the second centrifugation, 2.5 mL of plasma at the bottom of each tube was preserved and considered as PRP, whereas the supernatant (above 2.5 mL) was considered as platelet-poor plasma. At the end of the two centrifuges, 5 mL of autologous PRP was obtained and infused immediately after preparation.

Mares were randomly assigned to Control and PRP infusion cycle in a crossover study. Mares received only one infusion treatment per estrous cycle. Intrauterine infusion was performed using an artificial insemination pipette into the body of the uterus in the following groups: 1) PRP: intrauterine infusion of 5 mL of autologous PRP; 2) Control: intrauterine infusion of 5 mL of Lactated Ringer's solution. Within 24 h of ovulation induction followed by intrauterine infusion, the mares were inseminated with fresh semen.

Artificial insemination

Semen was collected from an 11-year-old Quarter Horse stallion, with an artificial vagina and a restrained estrus mare. After collection, the semen was evaluated for motility, vigor, and sperm concentration and diluent was added (Botusêmen - Botupharma®-São Paulo, Brazil) until $50x10^6$ sptz/mL. Insemination was performed with pipette inserted in the body of the uterus, with fresh semen, with motility ≥ 70% and a total concentration 500×10^6 motile sperm.

Ovulation

Within 48 h of insemination, the mares were evaluated using transrectal ultrasound to verify the presence of a corpus luteum and categorized as ovulated.

Embryo collection and evaluation

Eight days after ovulation (day 8), embryo recovery attempt was performed through transcervical uterine lavage and evaluated as described by MCKINNON & SQUIRES (1988) and MCCUE et al. (2009), in terms of size (microns), stage of development (morula, initial blastocyst, or expanded blastocyst), and quality (excellent, good, poor, and degenerated).

Statistical analysis

Qualitative variables are presented as absolute and relative frequency distributions using measures of central tendency and variation. The Shapiro-Wilk normality test was performed. Student's t-test was used to compare groups (PRP × control). The number of embryos recovered was considered the dependent variable. Values are presented as mean ± standard deviation (SD). Five percent was set to reject the null hypothesis. Statistical processing was performed using GraphPad Prism 9® software, version 9.0.0, for Windows (Dotmatics, San Diego, CA, USA).

RESULTS

A total of 49 embryo recovery attempts were performed from the older donor mares (Control, n = 23; PRP, n = 26). In the Control group, five embryos were recovered from 23 uterine flushes, with a recovery rate of 21.7%; while in the PRP group, nine embryos from 26 flushes, with 34.6% recovery rate (Figure 2; Table 1). Intrauterine PRP infusion in older embryo donor mares significantly increased the number of recovered embryos (P = 0.04). No adverse effect was observed in mares during experimental period.

In endometrial histological classification, most mares were classified as grade IIB (55.6%) or IIA (33.3%), followed by grades I (5,6%) or III (5,6%) (Table 1). Regarding endometrial histological

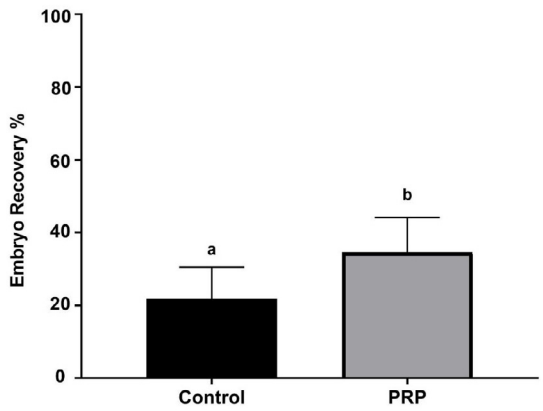

Figure 2 - Embryonic recovery rate in older embryo donor mares, according to Control and Platelet-Rich Plasma (PRP) group.

*Letters a, b indicate significant difference.

classification and embryo collection, in mares with grade IIA endometrium (mild fibrosis) significantly greater embryonic recovery was observed in the PRP group (40%) than in the Control (0%) (P = 0.0448) (Figure 3). In mares with histological classification IIB (moderate fibrosis), there was no significant difference between the PRP (26.6%) and Control (30.7 %) groups (P = 0.8190) (Figure 4).

In cytological exam, most mares presented negative cytology, in both the PRP and Control (80.6 and 69.6%, respectively), followed by severe (19.6 and 21%, respectively), moderate (0 and 4.3%, respectively), and mild (0 and 4.3%, respectively), regardless of positive or negative embryo recovery (Table 1). There was no significant difference between the groups and cytological evaluation.

All embryos collected from both infusion groups were classified as an expanded blastocyst developmental stage and excellent quality. The average embryo size in the Control was 207.5 µm and in PRP 174.3 µm, with no significant difference between groups in terms of embryonic size (P = 0.3709).

DISCUSSION

Several studies have already described the use of PRP in mares for reproductive purposes (COLOMBO et al., 2022; DAWOD et al., 2021; GHALLAB et al., 2023; REGHINI et al., 2014; SEGABINAZZI et al., 2017; 2021); however, no studies have addressed the intrauterine infusion of PRP in embryo donor mares aged > 15 years, which is considered advanced reproductive age. In the present study, advanced age donor mares infused with 5mL of PRP at the moment of ovulation induction showed significantly higher embryonic recovery, with an average of 34.6%, as opposed to 21.7% observed in the Control group.

The average embryonic recovery in young mares is 85% (2~4 years), in adults is 64.4% (4–18 years old), and in older mares is 24.1% (>18 years old) (SQUIRES & SEIDEL, 1995). Despite differences in age classification, our mean findings of embryo recovery in the Control group (21.7%) were slightly lower than those reported for older mares (24.1%) (SQUIRES & SEIDEL, 1995). The PRP group (34.6%) presented a 1.6 × higher average than the Control, indicating a positive effect of PRP infusion on embryonic recovery.

In the present study, no adverse effects associated with the intrauterine infusion of PRP were recorded, as described in the literature, including anaphylactic shock, systemic changes, severe uterine inflammatory reactions, and complications related to venipuncture (PASCH et al., 2021).

Studies suggested that PRP mitigates the inflammatory reaction in mares susceptible to persistent post-breed endometritis by reducing endometrial polymorphonuclear neutrophils and inflammatory cytokines, accumulation of intrauterine fluid, and reduction of positive bacterial culture (REGHINI et al., 2014; SEGABINAZZI et al., 2017; 2020; 2021) Other authors evaluated the intrauterine infusion of 20 ml of PRP and concluded that the treatment reduced the inflammatory response after mating, especially in mares classified as susceptible to endometritis (REGHINI et al., 2014; SEGABINAZZI et al., 2021). In the present study, the improved results observed with PRP could be associated with enhanced uterine clearance following artificial insemination, considering that older mares exhibit disturbances in the post-breeding uterine inflammatory response. It is worth noting that positive results were achieved with the infusion of a lower dose of PRP (5 mL), which led to higher embryo recovery rates. This lower volume also translates to reduced material costs and shorter preparation time (fewer tubes or bags to handle), making the procedure more practical for field application.

Uterine cytology can be performed under field conditions to detect the presence of endometritis at a practical and low cost (RIDDLE et al., 2007). In the present study, most older mares presented negative cytology results (69.5% Control; 78.6% PRP). In the

Table 1 - Results of embryo recovery, uterine cytology, and endometrial classification in older donor mares (n = 18) treated with uterine infusion of Platelet-Rich Plasma (PRP) or Control group.

Mare	Group	Embryo recovery	Uterine citology	Endometrial classification
1	Control	-	Negative	IIB
	PRP	-	Negative	
2	Control	+	Negative	IIB
	PRP	-	Negative	
3	Control	-	Severe	IIB
	PRP	-	Negative	
4	Control	+	Negative	I
	PRP	-	Negative	
5	Control	-	Severe	
	Control	-	Moderate	IIA
	PRP	+	Severe	
6	Control	-	Negative	
	PRP	-	Negative	IIB
	PRP	+	Negative	
7	Control	-	Severe	
	Control	-	Negative	IIB
	PRP	+	Severe	
	PRP	-	Negative	
8	Control	+	Mild	
	Control	-	Negative	IIB
	PRP	-	Severe	
	PRP	+	Negative	
9	Control	-	Negative	
	Control	-	Negative	IIA
	PRP	-	Severe	
	PRP	+	Negative	
10	Control	+	Negative	IIB
	PRP	-	Negative	
11	Control	-	Severe	
	PRP	-	Severe	IIA
	PRP	-	Negative	
	PRP	+	Negative	
12	Control	-	Negative	IIA
	PRP	-	Negative	
13	Control	-	Negative	IIA
	PRP	+	Negative	
14	Control	+	Negative	
	PRP	-	Negative	IIB
	PRP	+	Negative	
15	Control	-	Severe	IIB
	PRP	-	Negative	
16	Control	-	Negative	IIB
	PRP	-	Negative	
17	Control	-	Negative	
	PRP	-	Negative	IIA
	Control	-	Negative	
	PRP	+	Negative	
18	Control	-	Negative	III
	PRP	-	Negative	

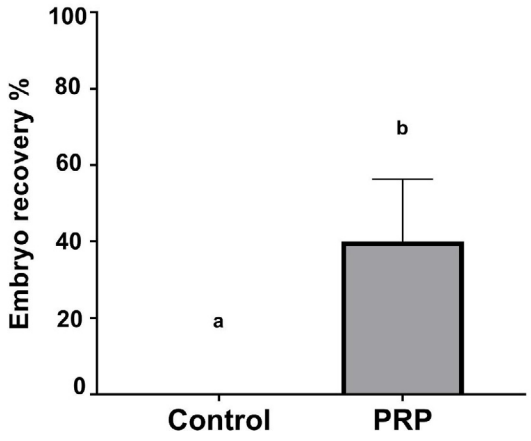

Figure 3 - Embryonic recovery rate in older donor mares with grade IIA endometrial histological classification, according to Control and Platelet-Rich Plasma (PRP) group.

*Letters a, b indicate significant difference.

Control, 21% of mares presented severe cytology and no embryos were recovered. In contrast, 21.7% of PRP mares also presented severe cytology, with embryos recovered in 40% of lavages, suggesting a beneficial action of PRP in embryo recovery.

The causes of reproductive history in advanced aged mares include a low embryonic recovery rate, uterine and tubal changes, increased rates of early embryonic loss (BALL et al., 1989), oocyte disorders, ovulation (CARNEVALE & GINTHER, 1995), and hormonal failures, all of which may occur concomitantly with endometritis (GOMES & GOMES, 2008). Endometrial fibrosis and the presence of glandular nests are associated with advanced reproductive age and are caused by degenerative changes (MORRIS & ALLEN, 2002). This condition is correlated with increasing age; and consequently, a decrease in reproductive capacity (DOIG et al., 1981; GRÜNINGER et al., 1998).

There was a higher prevalence of moderate to severe degenerative changes in mares over 20 years of age on endometrial histopathological evaluation than in younger mares (KABISCH et al., 2019). Most endometrial biopsies from mares over 16 years of age had histological classification grades IIA and IIB (WAELCHLI, 1990). Corroborating these findings, our results indicated that the majority (total of 93.8%) of endometrial biopsies were grades IIA (36.7%) and IIB (57.1%).

Regarding the endometrial biopsy classification, grade IIA includes cases with mild and scattered inflammation, mild fibrosis, endometrial atrophy, and a 50-80% estimated chance of pregnancy. While grade IIB corresponds to moderate and dispersed inflammation and moderate fibrosis, with a 10-50% chance of pregnancy, according to Snider's classification (SNIDER et al., 2011). In the present study, mares with grade IIA endometrial classification infused with PRP (40%) achieved a significantly higher number of embryonic recoveries than the control (0%) ($P = 0.0448$). Considering that they were older mares, embryonic recovery in the PRP group was close to the lower limit of pregnancy reported by SNIDER et al. (2011), PRP infusion significantly improved the recovery rate.

In the present study, all embryos collected from older mares showed excellent grades on the scale of 1-4 (MCCUE et al., 2009). This finding agrees with reports by other authors, in which most equine embryos were classified as good to excellent (MCKINNON & SQUIRES, 1988; PANZANI et al., 2014; SQUIRES et al., 2003). The main explanation is related to the physiology of embryonic transport, in which unfertilized oocytes, poor and degenerate embryos do not reach the uterine lumen and remain in the uterine tubes (MCCUE et al., 2009).

In equine breeding programs, it is estimated that up to 25% of mares are ≥ 16 years of age, when fertility is declining (ALLEN & PYCOCK, 1988). In embryo transfer programs, donor aged mares are

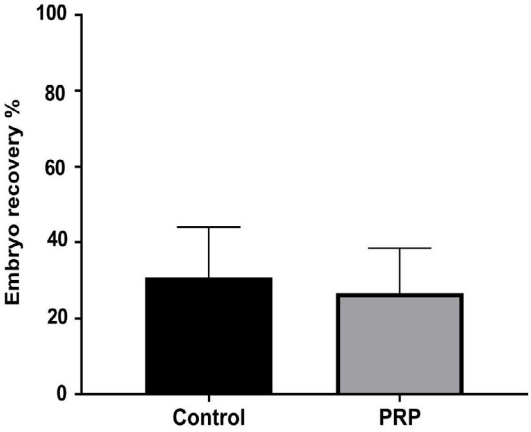

Figure 4 - Embryonic recovery rate in older donor mares with grade IIB endometrial histological classification, according to Control and Platelet-Rich Plasma (PRP) group.

common. In the present study, mares (n = 18) were aged between 18~25 years, and represented 28% of the stud breeding stock (n = 63). The 18 mares had already been discarded from breeding because of their advanced age and lack of embryonic recovery in the last two breeding seasons. Despite the limited number of old mares in the stud farm, consequently the limited number of embryos, intrauterine infusion of PRP provided a new opportunity for embryo recovery in older females with a history of unsuccessfully attempts. Further research may be necessary, including studies with larger sample sizes, investigations into fluid accumulation and clearance following artificial insemination, and evaluations of post-transfer pregnancy rates.

The costs of foal production through embryo transfer are high, and in the cases of subfertile and/or older mares, this cost increases exponentially, whereas the chance of success decreases (GOMES & GOMES, 2023). Research that encompasses viable alternatives to improve the efficiency of embryonic recovery in older mares is extremely important, both from an academic point of view, aiming to better understand the pathophysiology, and from an economic point of view, enabling for professional use under field conditions.

CONCLUSION

Intrauterine infusion of autologous platelet-rich plasma at the time of ovulation induction in older embryo donor mares significantly improved embryo recovery rates. PRP represents a promising therapeutic strategy to enhance reproductive efficiency in advanced-age mares and is feasible for use under field conditions.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

PSB conceived the investigation, methodology and writing the manuscript. VANS, MAT and PSBZ performed data and experimental validation. FCCS and AJOS performed methodology, conceptualization, review and editing.

BIOETHICS AND BIOSECURITY COMMITTEE APPROVAL

The animal study protocol was approved by the Ethics Commission on Animal Use (CEUA) of the Universidade Federal de Roraima (UFRR) under protocol #006/2021.

DATA AVAILABILITY STATEMENT

Raw data is available directly with the author.

DECLARATION OF USE OF ARTIFICIAL INTELLIGENCE

Artificial intelligence was used to assist language correction.

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