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Original Research

Reproductive Characteristics in Old and Young Subfertile Mares: Are They Really Different?

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ABSTRACT

Physiological and pathological mechanisms that determine subfertility (pregnancy failure, irregular cycles, and abnormalities in reproductive tract) in old mares (OM) have being studied by many authors. However, some young mares also share this reproduction condition although no previous reports have being published. We decided to investigate reproductive parameters of young subfertile mares (YSM) in order to understand the basis of their reproductive behavior. Forty-nine subfertile mares were classified and separated into 2 groups: YSM (3–10 years old; n = 28) and OM (13–23 years old; n = 21). Different number of cycles (1–8) was used for data analysis on the embryo recovery rate (ERR), interovulatory interval (IOI), multiple ovulation rate (MOR) and plasmatic progesterone. Embryo quality was evaluated by gene expression through RNAm analysis. Effluent samples were taken for bacteriological and cytological evaluation and endometrial biopsies were performed to evaluate the presence of inflammatory cells and endometrial progesterone receptors (PR). There was no significant differences in ERR ($P = .1230$) on the percentage of each embryonic stage found on the different days of flushing ($P > .05$); on embryo gene expression ($P > .05$); on MOR ($P = .1218$); and on plasmatic progesterone at day 8 PO ($P = .1230$). However, differences were found on the percentage of positive cytologies ($P = .0122$) and bacteriological cultures ($P = .0023$); the amount of mononuclear cells in biopsies ($P < .05$) and distribution of PR on endometrial localization. In conclusion, YSM share some physiopathological mechanisms with OM that could explain their reproductive performance's similarities.

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1. Introduction

Horses are selected by their reproductive competence but mainly as a result of their athletic performance. This leads to aged animals remaining in reproductive commercial programs due to the high genetic value of their offspring. It has been estimated that 10%–25% of the overall population of broodmares are older than 16 years old (y.o.) [1]. Advanced maternal age is an important predisposing factor on the reduction of reproductive efficiency [2,3,4,5]. It is widely accepted that mare fertility begins to decline from 13 to 17 y.o. leading this category of animals to be referred to as subfertile [6,7]. A subfertile mare can be defined as a mare that has been inseminated or covered by a stallion of proven fertility more than three opportunities during the breeding season and did not get pregnant; has abnormalities in the reproductive tract; shows irregular cycles during the breeding season; or does not get pregnant during several reproductive seasons [8]. As well as in other mammalian females, reduced pregnancy and foaling rates can be originated from ovulatory failure, poor oocyte quality, early embryonic loss, and fetal death, among others [9,2,10].

As a result of a longer follicular phase [9], older mares have been associated with an increased interovulatory interval (IOI) and also with lower circulating estrogen and inhibin concentrations, if compared to younger mares [11].

Multiple ovulation rate (MO) is also influenced by the age of the mare [12]. The MO can be defined as the percentage of cycles with double or triple ovulation in relation to the total number of cycles. Older mares have a higher MO rate than younger ones [5,13]. The effect of age on MO may be driven by a gradual increase in the IOI and differences in concentrations of gonadotropins as the mare becomes older [9].

It is known that the main cause of reduced pregnancy and foaling rates in old mare (OM) is the poor quality of embryos and the resultant high embryonic death between fertilization and the first 2 weeks of pregnancy [14]. The presence of intrinsic defects in oocytes and/or embryos significantly contributes to a reduction in embryonic survival related to maternal age [15]. Possible causes of structural and/or functional alterations in oocyte competence that could impact on embryo development are, for example, the decrease in mitochondrial activity and an increased incidence of chromosome abnormalities in aged females [1,16]. Frequently, OM are considered as the unique subfertile category, but they are not the only ones with reproductive problems. In fact, some young mares have also low reproductive efficiency, although no previous experiments have reported reproductive parameters responsible for this performance. Moreover, most research works have described reproductive disorders in old mares compared with fertile young mares.

Taking into account the information regarding physiological and pathologic mechanisms by which OMs are considered subfertile [14,17,18,2,3,4,15,19], we decided to investigate reproductive parameters of young subfertile mares (YSM) in order to understand the basis of their reproductive behavior. We hypothesize that YSM share reproductive characteristics with old subfertile ones such us IOI duration, plasmatic progesterone concentrations, MO, effluent contamination, embryo recovery rate (ERR), and embryo quality. The main objective of this study was to compare these reproductive parameters between both categories.

2. Materials and Methods

This work was performed during three consecutive breeding seasons at a farm located in Argentina (latitude 37° 25′ 28″S; longitude 59° 14′ 37″W). Forty-nine subfertile mares of Silla Argentino breed between 3 and 23 y.o. were used as donors. All mares used in this study belonged to the farm and were diagnosed as subfertile based on their individual history. These animals were classified and separated into two groups according to their ages and reproductive history: YSMs (3–10 y.o; n = 28) and OMs (13–23 y.o; n = 21). The older group included mares that had normal reproductive parameters when they were young, but for some reason, they become subfertile and were excluded from the farm breeding program. Subfertile mares were defined as those with a previous history of pregnancy failure, endometritis susceptibility, abnormal vulvar conformation, irregular cycles, hemorrhagic anovulatory follicles, embryo loss after ET, or no previous history of gestation. Mares from 11 to 12 y.o. were not included in the analysis in order to generate a clear age gap between both experimental groups. Different number of cycles (1–8) were used for data analysis on the ERR, IOI, and MO rate. Stallions younger than 13 y.o (n = 6) of proven fertility were used as semen donors.

Mares were routinely examined by transrectal palpation and ultrasonography to assess ovarian follicular activity. Once follicles of 32–35 mm in diameter and uterine edema were detected, and mares were artificially inseminated with fresh semen. Ovulations were evaluated by ultrasonography at 24-hour intervals, and their number was recorded in each estrus cycle. Mares in which ovulation had not been detected were reinseminated 24 hours after ultrasonography. The IOI was defined as the days between two consecutive ovulations. The MO rate was calculated by dividing the number of cycles with two or more ovulation by the total number of estrous cycles.

2.1. Embryo Recovery and Quality Assessment by Gene Expression Evaluation

The mare's uterus was flushed between days 7 and 9 after ovulation with 2 L of sterile Ringer Lactate solution at 35°C, using an equine lavage catheter CH 32 (Minitube International). Prior to the flushing, effective cleanness was performed to the vulva and perineal zone of each mare; sterile gloves were used to perform the flushing, with the objective of reducing possible external contamination or iatrogenic endometritis. The effluent was collected through a sterile plastic closed circuit, connected to an embryo filter. The collected fluid was rinsed into a sterile petri dish, where embryos were searched using a stereomicroscope at 20×. The number of recovered embryos was recorded for each flush. A uterine flushing was considered positive when one or more embryos were found.

In all performed flushings, a sterile sample of the effluent was taken from the petri dish, for cytological and bacteriological evaluation. We believe this evaluation is useful and reliable for early detection of endometritis because the sample is taken under extremely cleanness conditions.

After uterine flushing, each donor was treated with 250 μg of cloprostenol (Estrumate, MSD, Argentina) to induce luteolysis. At the same time, a blood sample from jugular vein was obtained for progesterone plasmatic concentration analysis (radioimmunoassay, according to Ginther et al [20]). Endometrial biopsies were taken on alternating cycles to evaluate the amount and type of inflammatory cells and progesterone endometrial receptors. A small sample (1–2 cm) of endometrial tissue was taken with a sterile endometrial biopsy instrument and then placed into a fixative solution (10% formalin).

Embryos were washed three times with Lactated Ringers Solution at 35°C in a laminar flow hood and then classified by its developmental stage (morula, early blastocyst, blastocyst, and expanded blastocyst) and quality grade (1–4), according to the scoring system proposed by McKinnon and Squires [21]. A group of embryos was stored individually in 100 μL Trizol (Invitrogen, CA) at −20°C for real time- polymerase chain reaction (RT-PCR) to determine apoptotic and stress genes expression through RNAm analysis, according with Canepa et al (2011). Briefly, five embryos collected in different flushing procedures from each experimental group were homogenized with 3.5 μg of tRNA (as a carrier to increase the RNA concentration) (Invitrogen, Life Technologies, Carlsbad, CA) using a tissue homogenizer. Groups were formed with five embryos of similar developmental stage belonging to mares from the same age group. RNA purification was performed by using the Direct-zolTM RNA Mini Prep, according to manufacturer's instructions. mRNA was transcribed using Superscript II enzyme (Invitrogen, Life Technologies) following the manufacturer's instructions. After reverse transcription, all samples were diluted 1:10 and used as template. The RT-qPCR reactions were carried out with an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). Primer sequences were designed using Primer Express Software v3.0 (Applied Biosystems) (Table 1). Amplicons were 60–100 bp long, and all of them were included in a pUC 57 (2,710 pb) vector, replicated in E. Coli. A total of five cDNA dilutions (1×10^{-5}, 1×10^{-6}, 1×10^{-7}, 1×10^{-8}, 1×10^{-9}) were prepared in order to make the calibration curve. The reactions were carried out with SYBRGREEN PCR Master Mix (Invitrogen) as described previously [22]. To verify that the SYBR Green dye had detected only one PCR product, all the reactions were subjected to the heat dissociation protocol following the final cycle of the PCR [23]. All samples were tested against GAPDH as a reference gene, for data normalization. Each RT-PCR quantitation experiment was done in triplicates for three independently generated cDNA templates.

2.2. Effluent Evaluation

Effluent samples taken from the petri dish were centrifuged at 400 *g* for 10 minutes. Bacteriological cultures were performed by using the pellet, which had been incubated at 35°C during 48 hours in blood agar. Results were considered positive when bacterial development was found. Cytological studies were performed by taking a sample with a swab from the same pellet, which was rolled into a glass slide. Then, it was stained with a commercial hematoxylin-eosin kit (Color Fast Kit, Biopack, Argentina) and evaluated by two different operators (100×) to detect the presence of polymorphonuclear (PMN) cells and the percentage in relation to endometrial cells. Results were considered positive when inflammatory cells detected were more than 5% of the total cells counted in 10 fields.

2.3. Endometrial Biopsy Evaluation

2.3.1. Inflammatory Cells

After flushing in alternating cycles, endometrial biopsy samples were taken and stained with hematoxylin-eosin. Samples were observed at 100× by two different operators to evaluate the number of inflammatory cells (PMN and MN cells) in 20 fields of stratum compactum and stratum spongiosum.

2.3.2. Progesterone Receptors

Endometrial biopsies were processed by immunohistochemistry to evaluate the number of progesterone receptors (PR) on glandular and luminal epithelium, compactum and spongiosum stratum. Samples were fixed in paraformaldehyde, embedded in paraffin, and processed for immunohistochemistry. Histological samples were treated with sodium citrat 0.01 M, pH 6.0 for antigenic recuperation. A primary anti-PR antibody diluted 1:50 was used (Zymed cat # 18-0172, South San Francisco, CA). Negative control was performed by replacing primary antibody by phosphate-buffered saline. Endometrial samples from mares in estrus were used as positive control. The Universal LSAB kit/HRP (Dako, Carpinteria, CA) was used, and samples were stained with hematoxylin for contrast. Samples were observed by two different operators to detect the number of immunostained cells in 10 fields (100×) of each endometrial area. Ten cells were observed in each field, making a total of 1,300 cells observed per group. Cells with a clear brown staining were considered as positive.

2.4. Statistical Analysis

All statistical analysis was performed with SAS V9.3 software. Embryo recovery rate, effluent evaluation, and plasmatic progesterone concentration were analyzed by using PROC GENMOD procedure as a binomial distribution. The IOI differences were calculated by *t* test, and MO rates were analyzed by chi-square test.

Inflammatory cells in endometrial biopsies were compared by analysis of covariance procedure using a Poisson distribution, and the amount of PR in endometrial biopsies was analyzed by mixed logistic regression using a normal distribution.

Differences in embryo development were calculated by PROC GENMOD using a multinomial distribution and stress gene expression by ANOVA PROC GLM using a normal distribution.

Table 1
Gene expression evaluation: oligonucleotides used.

Gene	Oligonucleotide	Sequence (5′-3′)	Tm (°C)	Amplicon (pb)	Gene Bank Registered Number
GAPDH	Forward	GGTTGTCTCCTGCGACTTCAA	59	64	NM_001034034.1
	Reverse	AATGCCAGCCCCAGCAT	59		
Bax	Forward	GCGCATCGGAGATGAATTG	59	59	NM_173894.1
	Reverse	CCACAGCTGCGATCATCCT	58		
Caspasa-3	Forward	TACTTGGGAAGGTGTGAGAAAACTAA	58	71	NM_001077840.1
	Reverse	AACCCGTCTCCCTTTATATTGCT	58		
ATF-6	Forward	AAGCTGCTCCATTTCTCACCAT	59	65	NM_001075935.1
	Reverse	CTTGCCTTGTGTCAGCTTCAGT	58		
HSP70	Forward	CCATCTTTTGTCAGTTTCTTTTTGTAGTA	58	75	NM_203322.2
	Reverse	GGAAGTAAACAGAAACGGGTGAA	58		
BiP	Forward	GATTGAAGTCACCTTTGAGATAGATGTG	59	85	NM_001075148.1
	Reverse	GATCTTATTTTTGTTGCCTGTACCTTT	58		
PSMB5	Forward	GCTTCTGGGAGAGGCTGTTG	59	69	NM_001037612.1
	Reverse	CGGAGATGCGTTCCTTGTTT	59		

Abbreviations: ATF-6, activatingtranscriptionfactor 6; Bax, Bcl-2 associated X protein; BiP, immunoglobulin heavy chain binding protein; GAPDH, gliceraldehído-3-fosfato-deshidrogenasa (housekeeping); HSP70, heat-shock protein 70; PSMB5, protease.

3. Results

3.1. Embryo Recovery and Quality Assessment by Gene Expression Evaluation

A total of 337 flushings (177 from YSM and 160 from OM) were performed at days 7, 8, and 9 after ovulation. There was no significant differences in ERR between YSM and OM (44.63% and 35.63%, $P = .1230$, Table 2).

A total of 152 embryos (88 from YSM and 64 from OM) were recovered and evaluated. There were no significant differences between YSM and OM in the percentage of each embryonic stage on day 7 PO ($P = .8072$), day 8 PO ($P = .6429$), and day 9 PO ($P = .0888$) (Table 3). Data from quality grade showed that all the embryos recovered were from grade 1.

Results obtained after processing 72 embryos by RT-PCR (45 from YSM and 27 from OM) showed not significant differences in the expression of the studied genes ($P > .05$).

3.2. MO Rate and IOI

A total of 177 cycles from YSM and 160 from OM were evaluated for multiple ovulation rate, and 288 IOI (148 from YSM and 140 from OM) were analyzed. Multiple ovulation rate was similar in both groups ($P = .1218$) (Table 2). However, the overall mean IOI of YSM (17.28 ± 0.43 days) was shorter than in OM (19.04 ± 0.53 days) ($P < .0187$).

3.3. Plasmatic Progesterone

Overall, 209 blood samples (112 from YSM and 97 from OM) were analyzed by radioimmunoassay. There was no significant difference ($P = .1230$) between age groups on plasmatic progesterone concentration (Table 2).

Table 2
Multiple ovulation rate (MOR), embryo recovery rate (ERR), plasmatic progesterone, and interovulatory interval (IOI) in young subfertile mare (YSM) and old mare (OM).

Mares Group	MOR (%)	ERR (%)	Plasmatic Progesterone (nmol/L)	IOI (d)
YSM	44/177 (24.9)	79/177 (44.63)	39.48 (±2.03)	17.28[a] (±0.43)
OM	52/160 (32.5)	57/160 (35.63)	41.12 (±2.33)	19.04[b] (±0.53)

[a,b]Different superscripts indicate significant difference ($P < .05$).

3.4. Effluent Evaluation

Effluent evaluation showed significant differences on the percentage of positive bacteriological cultures ($P = .0023$) and positives cytologies ($P = .0122$) between groups (Table 4).

3.5. Inflammatory Cells in Endometrial Biopsies

A total of 82 samples (48 from OM and 34 from YSM) were evaluated. Endometrial inflammation, based on PMN counting, exhibited similar patterns in both experimental groups ($P = .69$). However, significant differences were found regarding MN, in which OM showed higher counting in *stratum* compactum ($P = .01$) and spongiosum ($P = .02$) (Table 5).

3.6. PRs in Endometrial Tissue

Samples (13 from YSM and 13 from OM) were analyzed by immunohistochemistry to evaluate the amount of cells that presented positive stain for PR. No differences between experimental groups were found in the total amount of PR positive cells ($P > .10$) although significant differences were detected on their endometrial distribution ($P < .01$). Luminal epithelium immunostaining was higher in OM ($P < .05$) than in the other localizations (Table 6).

Table 3
Percentages of embryos recovered from young subfertile mare (YSM) and old mare (OM), on day 7, 8, or 9 postovulation (PO).

Day of Flushing	Age Group	Embryo Stage n (%)				Total
		Morula	Early Blastocyst	Blastocyst	Espanded Blastocyst	
7	YSM	5 (13.2)	24 (63.2)	6 (15.8)	3 (7.9)	38
	OM	2 (12.5)	9 (56.2)	5 (31.2)	0 (0)	16
8	YSM	4 (10.8)	6 (16.2)	23 (62.2)	4 (10.8)	37
	OM	2 (8.3)	6 (25)	13 (54.2)	3 (12.5)	24
9	YSM	4 (10.8)	6 (16.2)	23 (62.2)	4 (10.8)	37
	OM	2 (8.3)	6 (25)	13 (54.8)	3 (12.5)	24

Table 4
Positives bacteriological cultures and cytologies in young subfertile mares (YSM) and old mares (OM) groups.

Age Group	Effluent Evaluation	
	Bacteriological Culture (Positive %)	Cytology (Positive %)
YSM	37/177 (19.77)[a]	23/177 (12.99)[a]
OM	61/160 (38.13)[b]	45/160 (28.12)[b]

[a,b]Different superscript indicates a significant difference ($P < .05$).

4. Discussion

In this study, for the first time, YSM were compared with OM in order to find physiological parameters that could explain their similar reproductive performance.

Our results showed that ERR between YSM and OM is similar. Previous research had shown that more embryos were recovered per attempt in young mares (62%) than in old ones (51%), although pregnancy rate per transferred embryo was similar at 15 days [24]. It is known that OM has lower reproductive efficiency in embryo transfer (ET) programs than young mares [24] and that the incidence of early embryonic loss between fertilization and approximately day 40 may be as low as 10%–20% in young mares to more than 70% in aged mares [15]. Also, fewer pregnancies were lost after ET from young mares (8%) compared with old ones (23%) [24] showing that not only the ERR rate is affected, but also embryo quality when comparing a fertile and a subfertile group. We observed no differences in embryo development in the different days of embryo collection within both age groups; neither difference was found in embryo gene stress. Taking into account these results, we assume that embryos from YSM and OM have similar quality.

In our work, we found that OM has approximately 1 day longer IOI on average compared with YSM, thus being consistent with other authors reports in which young fertile mares and OM were studied [9,25,26]. Altermatt et al [27] and Rambags [16] suggested that the lengthening of the estrous cycle in OM could be caused by a slower growth rate of the dominant follicle. It has also been observed that old mares have fewer ovarian follicles and lower concentrations of LH during the preovulatory surge [28]. Results showed no differences in MO between young subfertile and old mares (24.9% and 32.5%, respectively). Previous data had reported that aging was associated with an increased incidence of MO [12,29]. Furthermore, MO in young mares reported from other authors [12,29,30] is lower than MO from YSMs in this study. The mechanisms governing MO in the mare are not clear. In cattle, there appears to be no association between MO and increasing gonadotropins. Instead, it is suggested that MO may be due to: increased sensitivity of follicles to gonadotropins, decreased inhibition of subordinate follicles by the dominant follicle, or an increase in ovarian production of IGF1. Although the physiological base of MO within a population is unclear, it has been suggested that in humans, it could act as a "natural insurance" against fertilization failure and as a compensation mechanism of defective oocytes or embryos [31]. We suggest that MO as a compensatory mechanism to poor oocyte and embryo quality, which is found in old mares as reported by [9,32], could be happening in YSMs. However, more studies are necessary to confirm this.

Low plasmatic progesterone concentration has been defined as one of the causes of early embryo loss, being 4 ng/mL considered as the minimal plasmatic progesterone level for pregnancy maintenance in mares [33]. In our study, values found in YSM and OM were higher than 4 ng/mL, so a progesterone low concentration could be discarded, as a main factor responsible for their low reproductive performance.

Endometrial biopsy evaluation showed that there is a similar amount of PMN between OM and YSM in the endometrial localizations evaluated. However, differences were found in the number of MN in both localizations, showing that in OM, the amount of MN was higher than in YSM. This result agrees with other authors, who confirm that old mares are more susceptible to chronic endometritis than young ones [34,35,8]. The aging and parity have been associated with altered systemic immune response [36,37]. Older mares present several predisposing factors to develop persistent uterine infections after breeding. They are known to accumulate fluid in the uterus as a result of impaired clearance of inflammatory products, which is caused by reduced myometrial contractions, poor lymphatic drainage, a large, overstretched uterus, and cervical incompetence. Altogether, these are predisposing factors for persistent mating-induced endometritis [38]. However, Ball et al [39] reported that chronic inflammation is not responsible for early embryonic loss produced in the aged mares. Immunohistochemistry results demonstrated no differences between both groups in the amount of PR in endometrial biopsies at day 8 PO, but a different distribution between both was found. In YSM, the amount of PR

Table 5
Mean levels of polymorphonuclear (PMN) and mononuclear cells (MN) in stratum compactum and spongiosum in young subfertile mare (YSM) and old mare (OM).

Group	N° Cycles	Stratum	PMN	MN
YSM (n = 22)	34	Compactum	1.51	0.94[a]
		Spongiosum	1.26	0.24[c]
OM (n = 19)	48	Compactum	1.43	1.45[b]
		Spongiosum	0.83	0.38[d]
		Spongiosum	0.83	0.38[d]

[a,b,c,d]Within column, different superscript indicates a significant difference ($P < .05$).

Table 6
Percentage of immunostained cells for PR in endometrial localization in young subfertile mare (YSM) and old mare (OM).

Endometrial Localization	YSM (%)[d]	OM (%)[d]	*P* Value
Stratum compactum	28.7[a]	26.5[a]	.97
Stratum spongiosum	34.3[b]	29.8[ab]	.86
Glandular epithelium	30.8[ab]	33.2[b]	.68
Luminal epithelium	34.2[b]	41.5[c]	.47

Abbreviation: PR, progesterone receptor.
[a,b,c]Within column, different superscript indicates a significant difference (Holm–Tukey: $P \leq .05$).
[d] Percentages are expressed on 1,300 cells observed per group and localization.

found in all localizations was similar; however, a higher amount of PR was found in luminal epithelium from OM biopsies, compared with other localizations. Previous reports demonstrated that there is a loss of PR in uterus epithelium before embryo implantation in ovine [40], equine [41], porcine [42], and bovine [43,44]. It has been established that the loss of epithelial PR represents a key factor to allow gene expression of endometrial proteins that induce molecular changes necessary for embryo implantation [45,46]. Thus, it has been determined that the exposure of the endometrium to progesterone causes a decrease in the expression of epithelial cell mucin, which is necessary to allow embryo adhesion [45]. Taking into account all this data, the absence of the loss of PR in luminal epithelium in OM could suggest the cause of possible implantation failure.

The analysis of the effluents revealed significant differences between groups, both in the number of positive bacterial cultures and the number of positive cytologies. Old mare had 2.6 and 2.5 more chances of having a positive bacteriological culture and cytology, respectively. Our results agree with other reports [38,47] which indicate that OM is more susceptible to postcoital endometritis, probably caused by their lower capacity to resolve it compared with young mares [48]. In this point, our results showed that YSM has a similar behavior to fertile young mares in order to resolve the postcoital endometritis. We suggest that the subfertility of YSM is not associated with a higher probability of endometritis.

In conclusion, YSMs share some reproductive characteristics with OM. Among these, ERR and embryo quality could be considered as the main causes of their subfertile condition because both are related to poor oocyte quality. In OM, we found additional alterations related to uterine function (positive bacteriological culture of effluents, chronic endometritis, and differential expression of PR) which could be responsible for embryonic loss. This suggests that the aging process is not the only cause of subfertility in mares, and other factors may play an important role and contribute to the subfertility process.

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