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Evaluation of the reproductive and metabolic problems encountered during two breeding seasons in barren Arabian mares

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Abstract: The aim of this study was to investigate the reproductive problems and determine the effect of treatment method on the pregnancy and foaling rates of 27 barren Arabian mares. The mares were examined over two study seasons. Ultrasonography and gynecological examinations were performed for all mares that were unable to conceive during the previous season and mares for which pregnancy was not achieved even after mating with stallions three times during each season. Endometrial swab samples were also obtained for microbiological examination. Cytology and endometrial biopsy samples were obtained from all mares in the first study season. Endometrial biopsy samples were also obtained from the mares in the second study season that were not pregnant at the end of the season due to the applied treatment. *Klebsiella pneumoniae* (42.9%) was the most frequently isolated species. A cytology score of >0 caused a decrease in the foaling rate ($P < 0.07$). A statistically significant decrease was observed in the pregnancy and foaling rate of mares in Category (C) > I ($P < 0.05$). Therefore, positive cytology and biopsy results should be considered prior to application of treatment in order to increase the expected foaling rate in barren Arabian mares.

Key words: Mare, breeding, infertility, pregnancy rate, foaling rate

1. Introduction

Mares with infertility problems commonly encounter 3 problems: accumulation of intrauterine fluid during estrus, infection and/or chronic inflammation for a long period of time, and estrous cycle disorders (1). Ovarian activity disorders that may cause infertility should be evaluated, and pathological changes in the reproductive organs, such as intrauterine fluid accumulation, can be detected during an ultrasonographic examination (2). Endometritis, which is one of the major causes of infertility in mares, is caused by inadequate bacterial inhibition, spermatozoa removal from the uterus, and inflammatory exudates after breeding (3). Although clinical endometritis is diagnosed easily, uterine culture, cytology, and endometrial biopsy are important tools along with ultrasonography examination that enable diagnosis of subclinical cases of endometritis (3). Previous studies have suggested that only uterine cytology or bacteriology (4) or a combination of both (4,5) can be used to determine uterus inflammation. In addition, various studies have also shown that uterine cytology and bacteria collected at the beginning of the season (before breeding) might affect the success rate of pregnancy and foaling (4,6). Degenerative changes, which are determined by endometrial biopsy, are also associated with the diagnosis of subclinical endometritis in mares as well as treatment success (3).

According to 2013 Turkish Statistical Institute (7) data, of 136,000 horses in Turkey, approximately 16,000 mares, aged ≥3 years, are used as broods. In the past, Arabians and thoroughbreds, in particular, were bred for use as workhorses, whereas presently they are used as racehorses in Turkey (8). Limited data are available on the reproductive performance of Arabian horses (9). The aim of this study is to investigate the causes of infertility in mares that were unable to conceive in the current and previous season on a farm that housed purebred Arabian horses. This study also aims to evaluate the treatment outcomes performed for the suspected etiology.

2. Materials and methods

2.1. Animals

The Committee for Research and Animal Experiments of Afyon Kocatepe University approved the experimental protocol (registration number: 02-11). This study was

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performed on a horse farm that breeds Arabian horses in the city of Eskişehir in Turkey and comprised 27 barren Arabian mares, aged 4–21 years. The causes of infertility in the mares that were unable to reproduce during the two breeding seasons and the results of the treatment interventions were evaluated. The infertile animals in the first breeding season included mares that were not pregnant from the previous season (A, B, C, D, E, F, G, Y, Z) and those that were not pregnant despite three inseminations in the same season (H, I, J, K, L, M, N, O). The infertile animals in the second breeding season included mares tracked in the first season that did not become pregnant (C*, F*, Y*, Z*, K*, L*, M*, N*, O*) (*9 barren mares that were not pregnant during the first breeding season) and mares that did not achieve pregnancy despite three inseminations in the second season (P, Q, R, S, T, U, V, W, X, β).

2.2. Methods

Mares that had not foaled the previous year were evaluated at the beginning of the breeding season (February). The same examination procedures were applied to the mares that did not become pregnant after 3 inseminations during the breeding season. Clinical and microbiological examinations were performed on all mares during the first and second seasons. Cytology and endometrial biopsy samples were collected from all mares in the first season and analyzed. Although cytology samples could not be obtained from some mares in the second season because of noncooperation by owners, endometrial biopsy samples were obtained from all mares that were unable to conceive at the end of the second season.

Clinical assessment included evaluation of perineal conformation, vaginal speculum examination, cervical digital examination, rectal palpation, and ultrasound examination (10). The same operator graded the body condition score (BCS) assessment from 1–9 (11). The condition of the uterus and ovaries was evaluated by ultrasound examination (Hitachi EUB-405, finger probe, 5.0 MHz). Follicular development and ovulation were monitored by daily rectal ultrasonography (1,10).

Endometrial swabs were taken for microbiological and cytological examinations using double-guarded swabs introduced inside the uterus (Equi-Vet, Kruuse, Marslev, Denmark). Swabs were taken from the endometrium and retracted into the inner guard, and then withdrawn from the mare as previously described (12). Sterile swabs were transported to the microbiology laboratory in Stuart's transport medium (Oxoid Ltd., Basingstoke, Hampshire, UK) for bacteriological examination. The samples were cultured on Columbia blood agar (Oxoid Ltd.), eosin methylene blue (EMB) agar (Oxoid Ltd.), chromogenic candida (CC) agar (Oxoid Ltd.) and Rappaport Vassiliadis broth (RVB) (Oxoid Ltd.). While the petri dishes (blood agar, EMB agar, and CC agar) were incubated under aerobic conditions at 37 °C for 24–48 h, the RVB was incubated at 42 °C for 24 h. After incubation, each different colony was examined macroscopically (colony morphology and hemolysis) and microscopically (Gram staining). Each different colony was then subcultured on Columbia blood agar (Oxoid Ltd.), containing 7% sheep blood and tryptone soya broth (Oxoid Ltd.) for further characterization. Identification of microorganisms was performed using the conventional methods according to standard manuals (13). Antibiotic susceptibilities of the isolated bacteria were determined using the Kirby–Bauer disk diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (14). For this purpose, gentamicin (10 µg), trimethoprim/sulfamethoxazole (25 µg), ceftiofur (30 µg), oxytetracycline (30 µg), and penicillin G (10 U) antibiotic disks (Oxoid Ltd.) were used.

After the first swab, the second endometrial sample was collected using the same sampling method. The swabs were rolled onto clean glass microscope slides. Endometrial smears were fixed using an ether-alcohol solution and stained using the Papanicolaou method (15). For cytological assessment, the percentage of neutrophils (PMN%) was ascertained in the entire area of the slide, and the smears were graded as follows: 0 (0%–0.5% PMN), +1 (0.5%–5% PMN), +2 (5%–30% PMN), and +3 (>30% PMN) (16).

Endometrial biopsy samples were fixed in 10% phosphate-buffered formaldehyde solution for 24 h and processed using paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin stain for histopathological examination (15). Endometrial biopsy samples were scored histologically according to the Kenney and Doig scale (17).

Blood samples (10 mL) were collected from the jugular vein of Arabian mares with a history of reproduction problems. Serum was separated by centrifugation at 3000 × *g* for 10 min and stored at –20 °C. Fresh blood samples were also collected in ethylenediaminetetraacetic acid (EDTA) vacutainers, protected from light, and transported to the laboratory for vitamin E analyses. Serum glucose levels were determined using a glucose analyzer (Cobas Integra 800; Roche Diagnostics GmbH, Mannheim, Germany). The levels of serum insulin were measured using an electrochemiluminescence immunoassay (Roche Diagnostics). Selenium levels were measured by flameless atomic absorption (PerkinElmer 100; PerkinElmer Corp., Norwalk, CT, USA). Plasma vitamin E levels were measured using high-pressure liquid chromatography with ultraviolet detection at 290 nm.

According to clinical or laboratory findings, the appropriate and current treatment approaches were applied to barren mares. A uterine irrigation process was

performed with fluid containing dimethyl sulfoxide/saline solution for 3 days in mares with chronic endometritis. The appropriate intrauterine antibiotics were administered during the following 3 days after termination of this process according to antibiogram results (1). Cloprostenol sodium (263 µg, Estrumate, 263 µg/mL, Intervet) was administered along with uterine irrigation at intervals of 4–6 h after breeding to mares with >2 cm intrauterine fluid collection, as ascertained by ultrasound examination during or immediately after estrus. Intramuscular dexamethasone (10 mg, Deksavet, 4 mg/mL, Interhas) was administered 24 h after mating (1–3,18). The mares with anovulatory follicles were administered 0.044 mg/kg Altrenogest (Altrogen-F, 3.3 mg/mL, Teknovet) orally for 11 days. Cloprostenol sodium injection (263 µg) was administered 1 day after the application (19).

Mares with a BSC of >7 (11) and blood serum insulin levels above 20 µU/mL (20) were diagnosed with insulin resistance and were administered 10 mg of levothyroxine sodium daily (Levotiron, 0.1 mg/tablet, Abdi Ibrahim) for 2 months. A single intramuscular injection of 10 mL of vitamin E-selenium solution (Injacom E-Selenium, 150 mg/mL vitamin E and 0.5 mg/mL selenium, Roche) was injected into mares with low vitamin E-selenium levels.

Caslick's operation was performed for mares with a vulva deformation and pregnant mares during the first control after the previous mating, in addition to the above-mentioned issues.

Two mating processes with fertile stallions were performed at intervals of 48 h for mares with ovarian follicles of >35 mm as detected by ultrasonography examinations. Human chorionic gonadotropin (hCG, 2500 IU) was administered to mares that were unable to ovulate 48 h after the last mating and with follicles of >50 mm. The day of disappearance of preovulatory follicles was considered as the day of ovulation (21). Ultrasonography was used to determine the pregnancy status at 15, 20, 35, 40, and 55 days after ovulation. Pregnancy loss between day 55 and foaling was determined by ultrasonography via rectum.

2.3. Statistical analyses

Pearson's chi-square test was used to determine the relationship between the bacteriology results and pregnancy-foaling rate, and Fisher's exact test was used to determine the effect of cytology and biopsy results on pregnancy and foaling rates. The foaling rate was used as the standard for determining the sensitivity, specificity, negative predictive value, and positive predictive value; these values were calculated according to the method described by Davis Morel et al. (4). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Clinical examination findings

Table 1 shows the distribution of various disorders identified as causes of infertility in all mares. The results of the clinical examination performed in mares that were not pregnant from the previous season in the first season, mares that were not pregnant despite three inseminations in the first season, mares tracked in the first season that did not become pregnant, and mares that did not achieve pregnancy despite three inseminations in the second season revealed that 2 out of 9 (22.2%), 5 out of 8 (62.5%), 4 out of 9 (44.4%), and 10 out of 10 (100%) mares, respectively, were normal. A single clinical finding was detected in 4/9 (44.4%), 3/8 (37.5%), 4/9 (44.4%), and 0/10 (0%) mares, respectively, and combined clinical findings were detected in 3/9 (33.3%), 0/8 (0%), 1/9 (11.1%), and 0/10 (0%) mares, respectively. The pregnancy rates in mares with normal clinical examination, with a single clinical finding, and with combined clinical findings were 14/21 (66.7%), 5/11 (45.5%), and 1/4 (25%), respectively, whereas the observed foaling rates were 12/21 (57.1%), 4/11 (36.4%), and 1/4 (25%), respectively.

3.2. Microbiology findings

Bacterial growth was detected in 21 (58.3%) out of 36 endometrial swab samples obtained in 2 years from mares. A single bacterial species was isolated from 17 swabs in which bacterial growth was detected, whereas mixed growth was observed in 4 samples. *Klebsiella pneumoniae* (n = 9/21, 42.9%) was the most frequently isolated bacteria, followed by *Staphylococcus aureus* (n = 2/21, 9.5%) and *S. aureus* + *Escherichia coli* (n = 2/21, 9.5%). The pregnancy rates achieved in mares with no growth, single isolate, and mixed isolate swab samples were determined as 66.7%, 41.2%, and 75%, respectively, and the foaling rate was determined to be 46.7%, 41.2%, and 75%, respectively. Although the pregnancy and foaling rates of mares with a single isolate were observed to be low compared to the others, the difference was not statistically significant ($P > 0.05$) (Table 2). The results of the Kirby–Bauer disk diffusion test showed that gentamicin was the most effective antibiotic (92%) against the factors isolated from endometrial swabs in mares, followed by trimethoprim/sulfamethoxazole (88%), ceftiofur (79%), oxytetracycline (54%), and penicillin G (29%), respectively.

3.3. Cytology and histopathology findings

Seventeen endometrial swabs and 24 endometrial biopsy samples obtained from mares were evaluated. Out of the obtained endometrial swab samples, 10 (58.8%) showed a score of +1 or above (Table 3). The pregnancy rates achieved in mares categorized with an endometrial swab score of 0 and >0 were 71.4% and 30%, respectively (Table

Table 1. Clinical, microbiological, cytological, and histological findings in mares used in the study with pregnancy rates and foaling rates achieved because of the applied treatments.

Rectal palpation, ultrasonography, and equine metabolic syndrome findings	Mares	Bacteria	Cytology score	Biopsy score	Pregnancy rate	Foaling rate
Normal clinical examination	A B H J L M O F* L* M* O* Q P R S W T U X V β	(*S. aureus*, coagulase-negative staphylococci) (*S. aureus*) (-) (*S. aureus*, *E. coli*) (*K. pneumoniae*) (*K. pneumoniae*) (-) (*K. pneumoniae*) (-) (*S. equi* subsp. *zooepidemicus*) (*K. pneumoniae*) (-) (-) (-) (*S. dysgalactia* subsp. *equisimilis*) (-) (-) (*K. pneumoniae*) (-) (*Staphylococcus schleiferi*) (-)	(0) (0) (0) (0) (0) (+3) (+1) (NS) (NS) (NS) (NS) (NS) (NS) (NS) (NS) (NS) (NS) (NS) (NS) (NS) (NS)	(I) (I) (I) (I) (I) (IIB) (IIA) (III) (NS) (NS) (IIA) (NS) (NS) (NS) (NS) (IIB) (NS) (NS) (NS) (III) (NS)	(+) (+) (+) (+) (-) (-) (-) (-) (+) (+) (-) (+) (+ED) (+) (+) (-) (+AB) (+) (+) (-) (+)	12/21
Inadequate uterine drainage	D G	(-) (*S. aureus*)	(+2) (+1)	(IIA) (IIB)	(+AB) (+)	1/2
Chronic endometritis (pyometra)	F	*Candida* spp.	(+2)	(IIB)	(-)	0/1
Uterine cysts	Y I K N Y* K* N*	(*S. aureus*, *E. coli*) (*Pseudomonas* spp.) (CNS) (-) (*K. pneumoniae*) (-) (*K. pneumoniae*)	(+3) (0) (+3) (0) (NS) (NS) (NS)	(IIB) (I) (IIB) (IIA) (NS) (NS) (IIA)	(-) (+) (-) (-) (+) (+) (-)	3/7
Insulin resistance	C*	(-)	(NS)	(IIA)	(-)	0/1
Insulin resistance + ovarian hematoma	Z*	(*K. pneumoniae*)	(NS)	(IIB)	(-)	0/1
Insulin resistance + anovulatory follicle + inadequate uterine drainage	C	(-)	(+2)	(IIA)	(-)	0/1
Insulin resistance + inadequate uterine drainage	Z	*K. pneumoniae*	(+2)	(IIA)	(-)	0/1
Inadequate uterine drainage + defective ulvar conformation	E	(*S. aureus*, *S. equi* subsp. *zooepidemicus*)	(+2)	(IIA)	(+)	1/1

The names of mares are arranged in alphabetical order. *Nine barren mares that were not pregnant during the first breeding season. NS: No sample, AB: abortus, ED: embryonic death.

Table 2. Pregnancy and foaling rates achieved via bacteriology, cytology, and biopsy examination results of the mares.

	Bacteriology (no growth, single isolate, mix isolate)	Cytology score (PMN = 0, PMN > 0)	Biopsy score (C = I, C > I)
Pregnancy rate (%)	10/15[a] (66.7%), 7/17[a] (41.2%), 3/4[a] (75%)	5/7[a] (71.4%), 3/10[a] (30%)	5/6[a] (83.3%), 3/18[b] (16.7%)
	P = 0.236	P = 0.153	P = 0.007
Foaling rate (%)	7/15[a] (46.7%), 7/17[a] (41.2%), 3/4[a] (75%)	5/7[a] (71.4%), 2/10[a] (20%)	5/6[a] (83.3%), 2/18[b] (11.1%)
	P = 0.578	P = 0.058	P = 0.003

Significant differences were observed between values indicated by different letters (a and b) within a column (P < 0.05).

2). The foaling rates of the mares with an endometrial swab score of 0 and >0 were determined to be between 71.4% and 20%, respectively; a value close to statistical significance was detected between the percentages (P = 0.058) (Table 2). Although one mare categorized with an endometrial swab score of +2 became pregnant, abortion occurred in the following period (Table 3). When the score results of 24 endometrial biopsy samples obtained from all mares were evaluated, the pregnancy and foaling rates of the mares with biopsy results categorized as Category (C) I and C > I were determined as 83.3% and 16.7% and 83.3% and 11.1%, respectively. A statistically significant difference was observed between the pregnancy and foaling rates of mares in Category (C) I and C > I (P < 0.05) (Table 2). Although pregnancy occurred in one mare with an endometrial biopsy result of CIIA, abortion was observed in the following period (Table 3).

When sensitivity, specificity, and negative and positive predictive values of bacteriology, cytology, and biopsy results were calculated considering the foaling rates, biopsy was observed to have the highest values and bacteriology the lowest (Table 4).

Table 3. Pregnancy and live foaling rates achieved according to the categorization of the results of endometrial swabs (0 (0%–0.5% PMN), +1 (0.5%–5% PMN), +2 (5%–30% PMNs), and +3 (>30% PMN)) and scores of endometrial biopsy.

	Cytological assessment				Endometrial biopsy score			
	0 score (n = 7)	+1 score (n = 2)	+2 score (n = 5)	+3 score (n = 3)	CI (n = 6)	CIIA (n = 9)	CIIB (n = 7)	CIII (n = 2)
Pregnancy rate (%)	5/7 (71.4%)	1/2 (50%)	2/5 (40%)	0/3	5/6 (83.3%)	2/9 (22.2%)	1/7 (14.3%)	0/2
Foaling rate (%)	5/7 (71.4%)	1/2 (50%)	1/5 (20%)	0/3	5/6 (83.3%)	1/9 (11.1%)	1/7 (14.3%)	0/2

Table 4. Sensitivity, specificity, negative predictive values, and positive predictive values of bacteriology (single isolate), cytology (swab score > 0), and biopsy (Category > I) in mares. The foaling rates were considered as the standard.

	Bacteriology (single isolate)	Cytology (swab score > 0)	Biopsy (Category > I)
Sensitivity	0.55	0.80	0.94
Specificity	0.50	0.71	0.71
Negative predictive value	0.46	0.71	0.83
Positive predictive value	0.59	0.80	0.89

4. Discussion

Studies conducted by Ulgen et al. (22) in mares of different breeds showed that fluid accumulation in the uterus is the most frequently observed clinical sign in mares (20.7%), followed by endometritis (15.8%) and endometrial cysts (12.7%). Vural et al. (23) reported that fluid accumulation in the uterus (33.3%) was the most commonly observed clinical signs in 12 purebred Arabian mares that were unable to foal regularly in the last 5 years. In the present study, uterine cysts were detected in 4 (14.8%), fluid accumulation in the uterus in 2 (7.4%), and chronic endometritis (pyometra) in 1 (3.7%). It was detected that clinical problems of the barren mares used in the study as material arose from accumulation of intrauterine fluid in the maximum rate, as reported by Ulgen et al. (22) and Vural et al. (23). Varied bacterial species are frequently isolated from the uteri of mares.

Although some researchers reported that *Streptococcus* group C (*Streptococcus equi* subsp. *zooepidemicus*, *Streptococcus dysgalactiae* subsp. *equisimilis*, and *Streptococcus equi* subsp. *equi*) (24) or beta-hemolytic *Streptococcus* (6) are the most frequently isolated from the uteri of mares with fertility problems, others reported that *E. coli* was isolated at higher rates (22,23). Large numbers of *K. pneumoniae* were isolated from 9 (25%) of the 36 swab samples obtained from the 27 mares evaluated in the present study over 2 years, unlike other studies. Various studies have reported that the isolation rates of *K. pneumoniae* from the uteri of mares with fertility problems vary between 3.8% and 23.2% (25,26). Although *K. pneumoniae* is phagocytosis-resistant (27), it is considered to be an important risk factor for the transmission of bacterial content in the penile region to the mare via stallions or in the semen used in artificial insemination (28). In this study, although bacteriological samples were not obtained from the genital area of the stallions, the reason for high incidence of *K. pneumoniae* may be due to venereal factors.

Pregnancy and foaling rates achieved based on bacteriological samples obtained from mares at the beginning of the season vary. Davies Morel et al. (4) reported that the foaling rate of mares from whom a single bacterial species was isolated from the first obtained swab samples before mating (51.0%) was significantly low as compared to the foaling rate of mares with no growth (74.2%) and mixed growth (71.7%). In the present study, although the foaling rate of mares from whom a single bacterial species was isolated from the obtained swab samples (7/17, 41.2%) was detected to be lower than the foaling rate of mares with no growth (7/15, 46.7%) and mixed growth (3/4, 75%), no statistically significant difference was observed. The reason for this was thought to be that the pure cultures obtained were associated more with acute metritis, as stated by LeBlanc (10) and Wingfield Digby and Ricketts (29). Mixed cultures (three or more organisms) are obtained because of general contamination (5). The pregnancy and foaling rates of mares from which mixed bacterial cultures were isolated were determined to be higher in the present study. Although Davies Morel et al. (4) detected the foaling rate of mares in which PMN was not present in the first swab samples obtained before mating to be 73.8%, Riddle et al. (6) determined the pregnancy rate achieved from 1503 mares in which no inflammation (0–2 neutrophils/field) was detected in cytological smears to be 59.1%. The foaling rates of mares with a score of 0 (74.1%) are similar to the findings of Davies Morel et al. (4), who reported that a cytology score of ≥2% PMN from the first swab samples obtained prior to mating significantly reduces the foaling rate. A cytology score of ≥0.5% PMN in the present study may reveal a different foaling rate ($P < 0.07$).

Although presence of infiltrative cells in the endometrium may be the main problem hindering pregnancy, it is reported that an increase in the degree of fibrosis can also prevent pregnancy continuation (30). An increase was observed in the degree of endometriosis in mares with an increase in age (31). Kenney and Doig (17) defined the expected foaling rates as a result of CI, CIIA, CIIB, and CIII categorization in the classification system, including four categories as >70%, between 50% and 70%, between 20% and 50%, and 0%, respectively (32,33). In Arabian horses in the present study, when the foaling rate was evaluated, the expected foaling rates were achieved in the CI and CIII categories, while lower than expected foaling rates were achieved in the CIIA and CIIB categories. The age, parity, reproductive status, clinical aspects, abnormal endocrinological causes, and syndrome cases were thought to be effective in addition to the biopsy scoring degree while obtaining this result (32).

Insulin dysregulation is important in equine metabolic syndrome. Insulin resistance may be caused by pathological conditions such as obesity, systemic inflammation, hyperadrenocorticism, and acromegaly, and these can occur physiologically during pregnancy and in stress conditions (34). According to our information, no study has investigated the relationship between infertility and insulin resistance. The cut-off values used in the diagnosis of hyperinsulinemia vary between 20 and 70 µU/mL in various studies (20,35). Although two mares with an insulin value of approximately 20 µU/mL in the present study were administered the required treatment interventions, pregnancy was not achieved in two seasons.

Therefore, it was determined that uterine infections, insufficient uterus drainage, uterine cysts, and metabolic factors play a role in the infertility of Arabian barren mares. Cytology and biopsy categorization scores in these mares affect the foaling rate. Treatment interventions for

etiopathogenesis may increase fertility in the facility and provide important economic inputs.

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