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Effect of Uterine Bacteriology and Cytology on Fertility in Thoroughbred Mares

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Abstract: This study was carried out to investigate the effect of uterine bacteriology and cytology on fertility in Thoroughbred horses during the period from April, 2008 to March, 2009 in Korea. About 91 strains were isolated from uterine culture swabs from 65 mares. The most common isolate was *Escherichia coli* (35 isolates, 38.5%) followed by *Klebsiella pneumoniae* (7 isolates, 7.7%), *Streptococcus equi* ssp. *zooepidemicus* (6 isolates, 6.6%) and organisms considered to be non-pathogens, other (43 isolates, 47.3%). Of the 65 cytological samples, 16 (24.6%) were positive for inflammation. The rate of pregnancy for normal, moderate and severe inflammation was 85.7, 42.9 and 22.2%, respectively. However, there are no significant relationship between bacteria and inflammation. In this study, researchers conclude that cytological examination of endometrium is a very effective method for a diagnosis of endometrial status. This research will contribute to the horse breeding industry including production of riding horses by artificial insemination as well as racing horses in Korea.

Key words: Uterine cytology, uterine bacteriology, fertility, Thoroughbred mare, horses, Korea

INTRODUCTION

Thoroughbred and Anglo-Arab horses that were imported form Japan and Australia have been used as racing horses in Korea since 1970's. Now-a-days, about 26,000 domestic horses was raised including 16,000 improved breed horses and 10,000 native breed horses. Among them, 2,300 Thoroughbred mares and 70 stallions was raised for breeding and 1,200 foals are bred every year (Choi *et al*., 2007).

Development and establishment of reproduction techniques are by far the most important aspect of producing high qualify breed stallions and mares. Unfortunately, there has been no significant study of horse reproduction in Korea. Moreover, self-sufficiency of domestic racing horse has reached 75% of all racing horses (Timoney *et al*., 1977).

Several factors are considered of importance for the breeding efficiency of Thoroughbred mares such as sire, age and reproductive status of mares and month of breeding (Bain, 1966). Uterine infections have long been recognised as one of the major causes of reduced fertility in the mares. Uterine infections often cause endometirtis. These infections are most often caused by opportunistic micro-organisms and a variety of species have been isolated (Brook, 1985). The acute endometrial inflammatory response after breeding is a predictable, physiologic event following the introduction of spermatozoa, bacteria and contaminants (Brook, 1985). Endometritis is most commonly associated with aerobic bacteria, sometimes anaerobic bacteria, pneumovagina, uterine fluid, spermatozoa and contaminant (McKinnon and Voss, 1993). The diagnosis of endometritis is critical in the veterinarian's attempt to treat infertility as soon as possible. In particular, examination of uterine status before breeding and appropriate treatment within a given time frame is the most important factor. Modalities that are available to diagnose endometritis include clinical examination, transrectal palpation and ultrasonography of the reproductive tract, vaginal speculum examination, uterine culture, cytology and endometrial biopsy (Parlevleit *et al*., 1977). Bacteriological culture of swabs from the surface of the endometrium has been used in the diagnosis of uterine infection (Dimmock and Edward, 1923). Although, the collection a samples from bacterial cultures is

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non-invasive and simple, the technique may provide false negative results (Pycock and Allen, 1990). Kudsen reported the potential value of endometrial cytology. Neutrophilis are very rarely seen in normal mares but can often be detected at the foal heat or the first estrus of the breeding season in maiden mares despite there being no detectable endometritis (Katila *et al*., 1988). The presence of abundant neutrophils in a cytological smear indicates that the mare has acute endometritis (Langoni *et al*., 1997; Roszel and Freeman, 1988). If only a few neutrophils are found, however various researchers (Liu and Troedsson, 2008) have applied semi-quantitative criteria to distinguish whether or not the mare has endometritis including the number of neutrophils per field. Because of the diagnostic nature of this technique, it is important that analysis of the endometrial swab accurately reflects the presence of endometrial inflammation. For reliable cytological results, a collection technique is required that yields many well preserved cells representative of a larger uterine surface. Endometrial cells for cytological evaluation may be collected in uterine aspirations, washings or by using guarded cotton swabs. A technique using the plastic cap of a guarded culture swab was also described. The importance of using a double-guarded swab has been recognised when collecting endometrial samples for bacteriology but there is little information on the importance of using guarded versus unguarded techniques to obtain representative swabs for cytological examination of the uterus (Javier *et al*., 2006; Liu and Troedsson, 2008).

Cytological examination is effective in determining uterine status from time to time. Moreover, it is an easy tool for quick diagnosis of uterine inflammation under practical field conditions. The objective of this study was to investigate how endometrial cytology findings related to uterine swab culture results and pregnancy rate.

MATERIALS AND METHODS

Experimental material: The samples were collected by using Equi-Vet uterine culture swab (Kruuse, Denmark) and BBL culture swab (Becton, Dickson and Co; Spark, USA) from 65 Thoroughbred mares that were repeat breeders or were suspected to have endometrial inflammation from April, 2008 to March, 2009 in the breeding season of the domestic horses in Korea.

The method of collecting samples involves inserting the vaginal swabs and uterine culture swabs into the genital tract for a minimum of 30 sec after taking away the tail or binding it with a band and washing the external genitalia with antiseptic solution. The collected sample was put into Thioglycolate broth and transported to the laboratory within 24 h to use in the study.

Isolation and identification of bacteria: The culture swabs of which the number of bacteria increased in Thioglycolate broth were inoculated to blood agar plate and MacConkey agar plate, respectively and cultivated at 37°C for 18~24 h. The colony was identified primarily through the colony form, Gram stain, OF test, catalase test, oxidase test, MacConkey agar growth test and lysine decarboxylase test and finally identified using Vitek (BioMerieux, France).

Endometrial cytology: Endometerial cytology was collected with Equi-Vet uterine culture swab (Kruuse, Denmark) when the mares were in estrus as previously described by Asbury (1984). The swabs were meared on a microscope slide (Marienfeld, Germany) for cytological analysis and fixed with a fixation solution (Hemal stain Co., USA).

Cytological specimens were stained with Diff Quick stain (Hemal stain Co., USA) and examined by microscopy (Axioscope A1, Zeiss, German). A minimum of 10 fields were evaluated on each slide. Findings were categorized as follows: normal cytology-epithelial cells and <2 neutrophils/field; moderate inflammation-epithelial cells and 2~5 neutrophils/field; severe inflammation-epithelial cells and >5 neutrophils/field.

Relationship between pregnancy rate, endometrial cytology and uterine culture: The study was conducted to evaluate the relationship between pregnancy rate, endometrial cytology and uterine culture. Also, researchers evaluated the relationship between cytology and results of breeding in barren mares, foaling mares and maiden mares of 2010.

RESULTS

Bacterial species isolated from 65 Thoroughbred mares: About 91 bacteria strains were isolated from 65 Thoroughbred mares as shown in Table 1. The most common isolate was *Escherichia coli* (35 isolates, 38.5%), followed by *Klebsiella pneumoniae* (7 isolates, 7.7%), *Streptococcus equi* ssp. *zooepidemicus* (6 isolates, 6.6%) and organisms considered to be non-pathogens, other (43 isolates, 47.3%).

Table 1: Bacterial species isolated from 65 Thoroughbred mares

Bacterial species	No. of bacteria isolated (%)
Escherichia coli	35 (38.5)
Klebsiella pneumoniae	7 (7.7)
Streptococcus equi ssp. *zooepidemicus*	6 (6.6)
Other	43 (47.3)
Total	91

Table 2: Relationship between pregnancy rates, endometrial cytology and uterine culture in Thoroughbred mares

Cytology* and culture	No. of mares	Pregnant (%)**
Normal		
No bacteria isolated	17	12 (70.6)
Bacteria isolated	32	30 (93.6)
Moderate inflammation		
No bacteria isolated	1	1 (100.0)
Bacteria isolated	6	2 (33.3)
Severe inflammation		
No bacteria isolated	1	1 (100.0)
Bacteria isolated	8	1 (12.5)
Total	65	51 (78.5)

*Normal, <2 neutrophils; Moderate inflammation, 2-5 neutrophils; Severe inflammation, >5 neutrophils; **Percentage of pregnant mares was calculated by dividing the number of pregnant mares by the number of mares

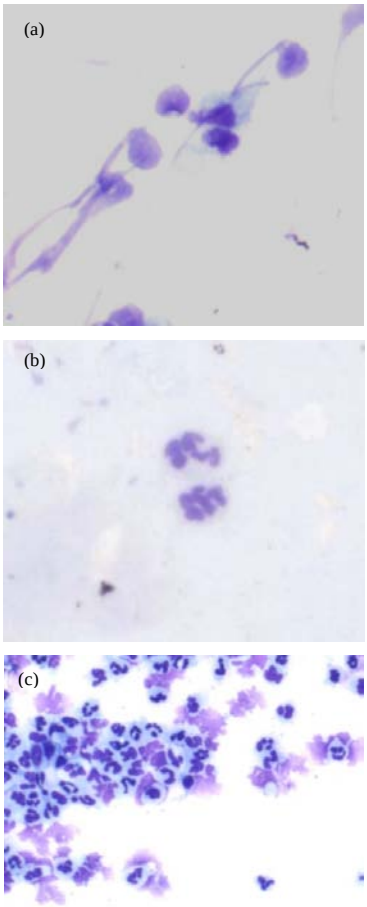

Fig. 1: Microscopic photographs of a cytology obtained from the endometrium. a) <2 neutrophils, mild inflammation; b) 2~5 neutrophils, moderate inflammation; c) >5 neutrophils, severe inflammation. Diff-Quick stain method (a-c, ×400)

Endometerial cytology: Of the 65 cytological smears with evidence of inflammation, 49 (75.3%) had normal status, 7 (10.8%) had moderate inflammation and 9 (13.9%) had severe inflammation were recovered as shown in Table 2 and Fig. 1.

Table 3: Relationship between bacteria isolated from uterine swabs and cytological findings in Thoroughbred mares

Bacterial species	No. of normal (%)	No. of moderate inflammation (%)	No. of severe inflammation*(%)
Eschericha coli	29 (54.7)	4 (26.7)	2 (8.3)
Klebsiella pneumoniae	2 (3.8)	1 (6.7)	4 (16.7)
Streptococcus zooepidemicus	0 (0.0)	2 (13.3)	4 (16.7)
Taylorella equigenitalis	0 (0.0)	0 (0.0)	1 (4.2)
Other	22 (41.5)	8 (53.3)	13 (54.2)
Total	53 (100.0)	15 (00.0)	24 (00.0)

*Normal, <2 neutrophils; moderate inflammation, 2-5 neutrophils; severe inflammation, >5 neutrophils

Relationship between pregnancy rate, endometrial cytological findings and uterine culture: The relationship between pregnancy rates, endometrial cytological findings and uterine culture are shown in Table 2 and 3. About 46 (70.8%) horses were isolated bacteria from 65 horses in this study. The pregnancy rate for 7 (10.8%) mares with moderate inflammation and 9 (13.8%) mares with severe inflammation was 3 (42.9%) mares and 2 (22.2%) mares, respectively. But the pregnancy rates for 49 (75.4%) horses with normal status was 42 (85.7%) horses.

However, there was no significant relationship between bacteria and inflammation. The most common isolated was *Eschericha coli* from normal horses whereas *Klebsiella pneumoniae*, *Streptococcus equi* ssp. *zooepidemicus* were isolated with high frequency from mares with moderate and severe inflammation.

DISCUSSION

There are several factors effecting on fertility in Thoroughbred mares. Among them, bacterial infection in the uterus is recognized as a major cause of reproductive failure in mares. These mares remain persistently infected and termed susceptible which predisposes other bacteria infection and decrease of resistance to infection.

Recently, Choi *et al.* (2007) reported that *Streptococcus* sp. (37.3%), *Staphylococcus* sp. (23.9%), *Bacillus* sp. (16.4%), *Corynebacterium* sp. (7.5%), *Enterobacter cloacae* (7.5%), *Escherichia coli* (5.9%) and *Bacteroides* sp. (1.5%) were detected from reproductive organs of normal mares. When mares were infected with Streptococcus, intrauterine fluid accumulated and the pH of endometrium was increased by super-antigens and streptokinase from Streptococcus, uterus responds quickly to an antigen with the release of PMN-chemotactic mediators (Timoney, 2004; Pycock and Allen,

1989). *Escherichia coli* infection mechanism of the uterus was not clear; however they made biofilm by polysaccharide and protein released from them and suppressed cell and humoral mediated immune response (Bettelheim, 1994).

Since endometritis is an inflammatory process, the diagnosis must be based on the presence of inflammation. The important thing for a diagnosis of endometritis is to confirm the presence of uterine inflammation. The best way to diagnose uterine inflammation is through a physical examination of the external and internal aspects of the reproductive system, endometrial cytologic evaluation and demostration of uterine fluid by ultrasonography (Parlevleit *et al*., 1977).

Bacteriological culture from swabs of the surface of the endometrium has been used in the diagnosis of uterine infection since the beginning of the past century (Card *et al*., 2004). Although, the collection of samples from bacterial culture is non-invasive and simple, the technique may provide false negative results. Later studies reported that mares with negative cultures may still have free uterine fluid and or debris on the external genitalia, consistent with endometritis. Prior to the 1980's, there are few reports on the cytology of equine reproductive tissue (Freeman *et al*., 1986; Solomon *et al*., 1972). Since that time, several reports have appeared that suggest a growing interest in this technique (Solomon *et al*., 1972; Wingfield and Richetts, 1982).

Riddle *et al*. (2007) reported that 230 (11%) mares were positive in a bacterial examination from endometrial cultures of 2044 mares. In pregnancy rates, 210 (10.3%) of mares with > 5 neutrophilis and 213 (10.4%) of the mares 2-5 neutrophilis in cytological examination had rates of 1.2 and 3.7%, respectively. However, 1621 (79.3%) of the mares 0-2 neutrophills in cytological examination had rates of 43.5%. A majority (45.6%) of the positive cultures had inflammation on a cytology smear. *Streptococcus equi* ssp. *zooepidemicus* was associated with more positive cytology results than coliforms. The percentage of cytological specimens graded as inflammatory varied among the microorganisms recovered. Isolation of beta-hemolytic Streptococcus, Staphylococcus or >2 organisms from the uterus were most commonly associated with cytological smears graded as severe whereas isolation of *Escherichia coli* and proteus were more likely no to have cytological evidence of inflammation.

Albihn *et al*. (2003) noted that non-hemolytic *Escherichia coli* isolated from 99 mares that had showed clinical signs of endometritis. They suggested that non-hemolytic *Escherichia coli* was associated the with pregnancy rate of mares. However, another report suggested that *Escherichia coli* did not have a significant relationship with pregnancy rate and induced opportunistic infection of other bacteria.

Michelle *et al*. (2007) noted that 25-60% of infected mares were not pregnant and it would cause big loss in the horse breeding industry. Low-volume uterine fluid was collected and examined for a diagnosis of endometritis from 308 barren mares. Sensitivity of bacterial examination and cytologiclal examination were 0.72 and 0.8, respectively by using low-volume uterine fluid. *Escherichia coli* was isolated primarily while the primarily pathogen of infertility was β-hemolytic Streptococcus. In this study, researchers determined the relationships between cytological findings and culture results from 65 mares. About 75.4% cytology samples were positive for inflammation. The pregnancy rate for 7 (10.8%) mares with moderate inflammation and 9 (13.8%) mares with severe inflammation were 3 (42.9%) and 2 (22.2%) whereas normal in 49 (75.4%) of the mares was 42 (85.7%) of pregnancy rates. Furthermore, the results of comparison of cytolgical status with bacteriological findings showed no significant relationship. *Streptococcus equi* ssp. *zooepidemicus* was isolated from 100% of mares with severe inflammation. However, 8.3% of *Escherichia coli* was isolated from mares with severe inflammation among 89.7% of isolated *Escherichia coli*. In addition, uterine cytology results were 3 (21.4%), 4 (28.6%) and 7 (50.5%) among barren mares with normal, moderate inflammation and severe inflammation, compared with 42 (89.3%), 3 (6.4%) and 2 (4.3%) among foaling mares with normal, moderate inflammation and severe inflammation.

Endometrial cytology and culture were diagnostics for identifying mares with endometritis and pregnancy rate in the study. These findings were similar to those of Riddle *et al*. (2007) who reported that normal in 72% of the mares in cytological examination had a 60% pregnancy rate whereas mares with cytological evidence of inflammation of the uterus had a decreased pregnancy rate with the lowest pregnancy rates in mares exhibiting severe inflammation. Researchers should further investigate collecting methods in cytological examinations which may prove to be more helpful as a clinical tool.

CONCLUSION

In this study, researchers believe that cytological examination of endometrium is a very effective method for determining endometrial status; however, it should be performed using more samples. This research will contribute to advances in the horse breeding industry in Korea including production of riding horses by artificial insemination as well as racing horses.

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