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Original article

Prevalence of subclinical endometritis in repeat breeding cows and mRNA expression of tumor necrosis factor α and inducible nitric oxide synthase in the endometrium of repeat breeding cows with and without subclinical endometritis

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Abstract

Information on the prevalence of subclinical endometritis and its mechanism in repeat breeding cows is very limited. The aims of this study were: a) to evaluate the incidence of this disorder with cytobrush cytology b) to analyze mRNA expression of tumor necrosis factor α (TNFα) and inducible nitric oxide synthase (iNOS) in endometrial biopsy samples collected from repeat breeding cows with and without subclinical endometritis.

Two experiments were carried out. In experiment 1, 112 (12.4%) repeat breeding cows (inseminated at least 3 times and not pregnant) were selected out of 902 cows from 8 dairy herds. Cytobrush cytology was performed on these cows, using the threshold of 10% PMNs in uterine smears. The results showed that 45 out of the 112 cows (40.2%) were diagnosed as having subclinical endometritis. In experiment 2, uterine biopsy samples were taken from repeat breeding cows with subclinical endometritis (n = 10) and without this disorder (n = 10). Using reverse transcription-PCR, the mRNA expression of TNFα and iNOS was determined. A statistically significant increase in expression of both substances was measured in the group of cows with subclinical endometritis ($p < 0.05$).

These results provide evidence for a high prevalence of subclinical endometritis in repeat breeding cows as well as the involvement of TNFα and iNOS pathways in the regulation of this pathological condition.

Key words: cows, repeat breeding, endometritis, mRNA expression, TNFα, iNOS

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Introduction

Repeat breeding continues to be a worldwide problem in dairy herds, where its prevalence has been reported to reach 30% (Bartlett et al. 1986, Stolla and de Kruif 1999, Gustafsson and Emanuelson 2002, Baranski et al. 2007, Yusuf et al. 2010). A repeat breeder cow is defined as a normally cyclic cow without any clinical abnormalities that is not pregnant after several successive inseminations (Zemjanis 1980). Under field conditions, repeat breeding is associated mainly with subclinical endometritis, delayed ovulation and *corpus luteum* deficiency, which cause failure of fertilization or embryonal mortality (Stolla and de Kruif 1999, Parkinson 2009).

During the last decade, subclinical endometritis and its impact on fertility have been intensively investigated. Subclinical endometritis is an inflammation of uterine mucosa in the absence of clinical signs of endometritis, which causes significant reduction of reproductive performance (Sheldon et al. 2009). Subclinical endometritis can be diagnosed by uterine biopsy, ultrasonography and endometrial cytology. Endometrial cytology can be collected by uterine lavage or with a cytobrush. The most reliable method for diagnosing subclinical endometritis is cytobrush cytology (Barlund et al. 2008). Endometrial samples are collected with a small brush and the percentage of polymorphonuclear neutrophils (PMNs) is calculated. To differentiate between cows with endometritis and healthy cows, different thresholds (5%, 10%, 18%) are used (Kasimanickam et al. 2004, Gilbert et al. 2005, Plöntzke et al. 2010, Baranski et al. 2012, Ghasemi et al. 2012). The prevalence of sublinical endometritis in various studies ranges from 20% to 90%, depending on the threshold used and the postpartum examination timing. Most studies have focused on the first two months after parturition. There are very few papers dealing with cytological diagnosis of subclinical endometritis around breeding. Kaufmann et al. (2009) demonstrated that cows with high proportions of endometrial PMN (> 15% PMN) 4 h after AI have a decreased first service conception rate. Salasel et al. (2010) used the lavage technique and showed high prevalence of subclinical endometritis in repeat breeding cows. Until now, there have been no studies performed to investigate the prevalence of subclinical endometritis diagnosed by the cytobrush technique in repeat breeding cows.

The uterus is routinely contaminated with bacteria in the early postpartum period (Noakes et al. 1991, Sheldon and Dobson 2004, Foldi et al. 2006, Singh et al. 2008). A high proportion of infected cows, irrespective of treatment, have a spontaneous resolution of endometritis at 4-6 weeks postpartum, whereas the remainder have persistent infection during the service period. These infections are believed to result in some damage to gametes and embryos, causing impaired fertility (Sheldon et al. 2009).

It has been documented that abnormal endometrial function and/or uterine mucosa damage as a consequence of infection may contribute significantly to repeat breeding incidence. Earlier studies on the relationship between subclinical endometritis and repeat breeding were performed using the endometrial biopsy technique (Hartigan et al. 1972, Seitaridis and Tsangaris 1973, Schmidt-Adamopolou 1978, Dogan et al. 2002). This method is obviously an essential component of the examination of cows with unexplained infertility, but it is costly, time consuming and not easily accessible under field conditions. Therefore, a simpler method such as uterine cytology emerges as a useful alternative.

Uterine cytology, which provides valuable information for diagnosis of subclinical cases, is based on the number of PMNs in relation to epithelial cells. Because the uterine influx of PMNs is the main element of the innate immune system involved in the resolution of infection, a rising count of these cells reflects the severity of local inflammation. In general, this defence system is activated in the presence of pathogen-associated molecules such as bacterial or viral LPS, DNA and lipids recognized by TLR-receptors (Herath et al. 2006, Singh et al. 2008, Sheldon et al. 2009). Engagement of these receptors initiates a signalling inflammatory cascade, stimulating production of many mediators (cytokines, chemokines, prostaglandins etc.), including tumour necrosis factor α (TNFα) and nitric oxide (NO). However, the regulation of endometrial immunity during different reproductive disorders is not fully known. Higher expression of some pro-inflammatory factor transcripts in the endometrium of cows with postpartal subclinical endometritis compared with healthy cows has been found (Gabler et al. 2009, Fischer et al. 2010, Ghasemi et al. 2012). Also, the association between immunological aspects of endometrial diseases and repeat breeding in cattle and other species is poorly understood. Limited information about these mechanisms is provided by studies performed on laboratory animals, for example enhanced mRNA expression of TNFα and nitric oxide synthase (iNOS) was found in the endometrium in mice and rats with early embryonic resorption (Haddad et al. 1995, 1997, Ogando et al. 2003).

Lack of such studies on cows as well the scarcity of information on the prevalence of cytological endometritis in repeat breeding cows encouraged us to undertake this study, whose aims were a) to evaluate the prevalence of subclinical endometritis with cytob-

rush cytology, b) to analyze the mRNA expression of TNFα and iNOS in endometrial biopsy samples collected from repeat breeding cows with and without CE.

Materials and Methods

Experiment 1. Prevalence of subclinical endometritis in repeat breeding cows

Animals

The study was carried out on 8 dairy herds comprising 902 Polish Holstein-Friesian cows, two to seven years old, with an average milk yield of 6000-9000 L. The cows were housed in loose barns and fed grass and maize silage, concentrates, and vitamin and mineral supplements. The total mixed or partial mixed ration feeding system was used. The cows that were inseminated at least 3 times and those still not pregnant were examined by vaginoscopy, rectal palpation and ultrasonography for reproductive disorders and determination of the estrus cycle phase. Out of the 902 cows, 112 (12.4%) clinically healthy, repeat breeding cows were selected for the study. They were at 170 ± 32 DIM.

Samples collection

Samples were collected from the cows in the luteal phase of the estrus cycle using the cytobrush method (Barański et al. 2010). In order to obtain samples from the cows, sterile brushes normally used for cytological examinations in human gynaecology were applied (Cervical Rambrush type I C, Shanghai International Holding Corp. GmbH, Germany). The brush was slid over the mandrel, and then the brush and the mandrel were inserted into a stainless steel catheter. To avoid vaginal contamination of the brush, the entire setup was inserted into a sterile glove for transrectal examination (Kruuse, Denmark). This setup was then inserted into the cow's genital tracts. For the catheter to pass through the cervix, the cervix was manipulated manually through the rectum. After the catheter had passed through the cervix, the glove protecting the catheter was punctured. The catheter was then moved up into the uterine horn. Once the catheter entered the horn, a sample was taken by pushing the brush out of the catheter and rotating it three times. The brush was then pulled back through the catheter and out of the cow. The material collected with the cytobrush was transferred onto a microscope slide by rolling the brush on the slide. The purpose was to determine the ratio of PMNs to epithelial cells.

Cytological examination

The smear was then treated with cytological fixative (Cytofix®, Samko, Poland). The preparations were stained by Papanicolau's method, and in each smear a hundred visible cells were examined with a light microscope and their proportion was calculated. The threshold of 10% PMNs was used for the diagnosis of subclinical endometritis (Kasimanickam et al. 2004). PMNs and epithelial cells were counted by a veterinary surgeon, who was blind to the study population.

Experiment 2. TNFα and iNOS mRNA expression in the endometrium of repeat breeding cows with and without subclinical endometritis

Animals

The study was carried out on one herd, composed of 210 Polish Holstein-Friesian cows. The cows were from 2 to 7 years old, with an average milk yield of 7000 L. The cows were kept in loose barns and fed grass and maize silage, concentrates, and vitamin and mineral supplements. The partial mixed ration feeding system was used. Ten repeat breeding cows with cytologically determined endometritis and 10 cows without it were included in the study.

Samples collection

Samples from the uteri for cytological examination were collected using the cytobrush method as described above. Endometrial biopsies were collected using an endometrial biopsy instrument (Hauptner, Solingen, Germany) as described previously (Bonnet at al. 1991). After cleaning the perineum and external genitalia, the biopsy instrument was introduced into the uterus guided by trans-rectal palpation. The biopsy was taken from the larger diameter horn. The tissue was placed immediately into a 1.5 ml tube containing preservative to maintain mRNA integrity, and transported to the laboratory.

RNA extraction

The tissues were homogenized in tubes containing Lysing Matrix D beads using MP FastPrep homogenizer. RNA was extracted using TRI Reagent® (T9424, Sigma-Aldrich) according to the manufacturer's instructions. Evaluation of RNA concentration was done spectrophotometrically (NANO Drop 2000,

Table 1. Gene transcripts, primer sequences, amplicon length and GenBank accession number.

Gene	Primer sequence	Amplicon length (bp)	GenBank accession no.
GAPDH (Bos taurus)	CACCCTCAAGATTGTCAGCA/ GGTCATAAGTCCCTCCACGA	103	BC102589
iNOS	GGTGGAAGCAGTAACAAAGGA/ GACCTGATGTTGCCGTTGTTG	230	AF340236
TNFα	CCGCATTGCAGTCTCCTACC/ TGGGTTCATACCAGGGCTTG	110	NM_173966.2

OD 260/280). One microgram of total RNA from each sample was reverse transcribed using a QuantiTest Reverse Transcription Kit (205311, Qiagen, Hilden, Germany) as described in the supplier's protocol. The cDNA generated was stored at – 20°C until use.

Real-time RT-PC

Real-time RT-PCR assays were performed in a 7900HT Real Time PCR System (Applied BiosystemsTM, Warrington, UK), using the default thermocycler program for all genes: 10 min pre-incubation at 95°C was followed by 40 cycles for 15 sec at 95°C and 1 min at 60°C. A further dissociation step (15 sec at 95°C, 30 sec at 60°C and 15 sec at 95°C) ensured the presence of a single product. In each real time assay, both target gene and HKG (housekeeping gene, GAPDH) were run simultaneously. All reactions were carried out in duplicate wells on a 96 well optical reaction plate (Applied Biosystems, ref. 4306737, UK) in 20 µl reaction volume: 9 µl water with forward primer (160 nM) and reverse primer (160 nM); 10 µl Power SYBER Green Master Mix (Applied Biosystems, Ref. 4367659, UK) and 1 µl of 4 tims diluted cDNA. The primer pairs are shown in Table 1.

For the relative quantification of mRNA expression levels (target gene versus housekeeping gene), miner software was used (http://www.miner.ewindup.info/version).

Statistical analysis

All data are shown as mean ± SEM. The statistical significance of differences in mRNA expression between both groups was analyzed using the one-tailed nonparametric Mann-Whitney test (GraphPAD PRISM, Version 4.00, GraphPad Software, San Diego, CA, USA). The levels of significance was set at $P < 0.05$.

Results

Experiment 1. Prevalence of subclinical endometritis in repeat breeding cows

Out of the 902 cows, 112 did not become pregnant after three inseminations, although they did not present any clinically detectable reproductive disorders. Thus, the percentage of repeat breeding cows was 12.4%. With the cytobrush technique, 45 (40.2%) of 112 clinically healthy repeat breeding cows were diagnosed as having subclinical endometritis (Fig. 1).

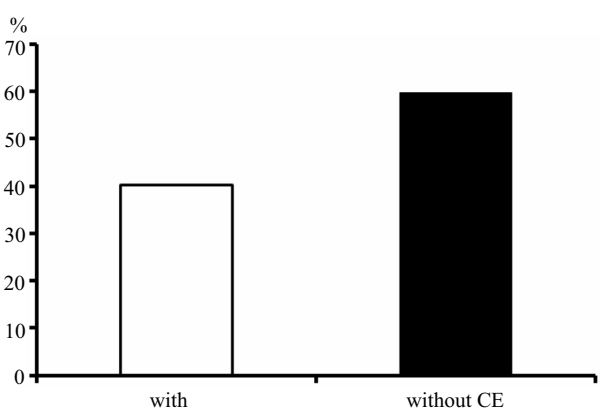

Fig. 1. Prevalence of cytologically determined subclinical endometritis (CE) in repeat breeding cows.

Experiment 2. TNFα and iNOS mRNA expression in the endometrium of repeat breeding cows with and without of cytologically determined subclinical endometritis

The mRNA expression of iNOS and TNFα was significantly higher ($p < 0.05$) in repeat breeding cows with subclinical endometritis than in those without it. A 2-fold higher iNOS mRNA expression and 3-fold higher TNFα mRNA expression was noted in endometrial samples obtained from cows with subclinical endometritis compared with samples from healthy cows (Fig. 2-3).

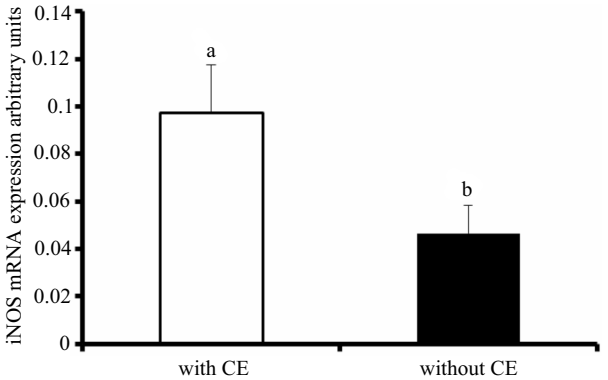

Fig. 2. Expression of iNOS mRNA in endometrium of repeat breeding cows with and without CE. a-b difference statistically significant at $p<0.05$.

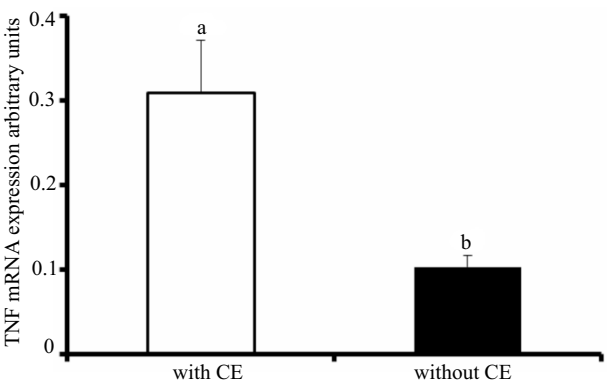

Fig. 3. Expression of TNFα mRNA in the endometrium of repeat breeding cows with and without CE. a-b difference statistically significant at $p<0.05$

Discussion

Repeat breeding remains one of the most widespread problems in dairy cows and is becoming more serious with modern breeding technologies (Parkinson 2009, Salasel et al. 2010). In the present study, the incidence of repeat breeding in dairy cows was 12.4%. This percentage is in agreement with data previously reported (Gustafsson and Emanuelson 2002, Yusuf et al. 2010), whereas a higher repeat breeding incidence of 24% in US dairy herds was reported by Bartlett et al. (1986).

The study revealed a high prevalence of subclinical endometritis in repeat breeding cows. This is one of the first attempts to investigate cytologically the prevalence of this disorder in cows inseminated unsuccessfully. To the authors' best knowledge, there is just one other similar report, by Salasel et al. (2010), who observed a slightly higher percentage of cows with this disorder (52.7% vs 40.2%). Such a small difference between the two studies can be associated with different study populations and different environmental factors to which the herds were exposed. Some researchers claim that the prevalence of this disorder in postpartal cows is a herd-specific feature (Gilbert et al. 2005, Barański et al. 2012). Because our study was conducted on 8 commercial dairy herds, our data reflect a real situation in the population of Polish dairy cows. However, the general results of both studies are in line and confirm a high proportion of repeat breeding cows affected by subclinical endometritis. It should also be stressed that the similar results obtained in the two studies were achieved with different diagnostic methods. Salasel et al. (2010) applied uterine lavage and a threshold of 3% PMNs, whereas we used the cytobrush technique and a threshold of 10% PMNs. Currently, cytobrush cytology is considered as a practicable method for monitoring the uterine health of cows without any clinical signs of endometritis (Kasimanickam et al. 2004, Barlund et al. 2009). In a comparative study by Barlund et al. (2009), the cytobrush technique was evaluated as more reliable owing to its superior repeatability.

In studies on subclinical endometritis, the threshold ratio used for diagnosis ranges from 5% to 18% PMNs, depending on the author and sampling time after parturition (Kasimanickam et al. 2004, Gilbert et al. 2005, Plöntzke et al. 2010, Baranski et al. 2012, Ghasemi et al. 2012). However, many authors believe that the threshold ratio used for differentiation of cows suffering from subclinical endometritis should be refined (Gilbert et al. 2005, Dubuc et al. 2010, Baranski et al. 2012).

In our study, in order to distinguish cows with and without subclinical endometritis, the threshold ratio of 10% was used. It is difficult to compare this limit with other studies because an optimum threshold ratio for repeat breeding cows has yet to be documented. Many reports found a threshold value of 5-10% PMNs as indicative of subclinical endometritis at about 60 days postpartum (Kasimanickam et al. 2004, Baranski et al. 2012). Moreover, Kaufmann et al. (2009) used a threshold value of 15% PMNs 4 hours after insemination in cows mated > 100 days after parturition and observed a lowered pregnancy rate in cows with both enhanced > 15% PMNs and lowered < 5% PMNs ratios. Considering these data as well as the fact that our research was conducted long after parturition, in which it was similar to the study cited above, it seemed reasonable to use the cut-off point of 10% for the group of repeat breeders.

To our knowledge, this study is the first attempt at investigating the prevalence of subclinical endometritis using the cytobrush method. We are able to show a relatively high proportion of repeat breeding cows affected by this disorder, which, irrespective of methodological differences, is in agreement with earlier studies based on endometrial biopsy. In those stu-

dies, the prevalence of subclinical endometritis in repeat breeders ranged from 40% to 90% (Hartigan et al. 1972, Seitaridis and Tsangaris 1973, Schmidt-Adamopolou 1978, Dogan et al. 2002). As mentioned earlier, the latest results obtained by uterine lavage and cytological examination (Salasel et al. 2010) were also similar to ours.

Many factors are suspected to be involved in repeat breeding, e.g. disturbed embryonic development, abnormalities of ovulation, heat stress, inadequate progesterone level and inflammation of the endometrium (Stolla and de Kruif 1999, Parkinson 2009). Obviously, this study concerns only some of the causes, related to the inflammatory processes in the uterus and their consequences. Gautam et al. (2010) reported that about a quarter of cows with postpartum clinical endometritis presented persistence or recurrence of endometritis during the breeding period.

Another interesting aspect of this study was the evaluation of the mRNA expression of TNFα and iNOS in the endometrial tissue of repeat breeding cows with and without subclinical endometritis. This experiment was performed as a study on the mechanisms contributing to embryonal mortality and repeat breeding simultaneously. Our results revealed that the expression of genes coding these factors was significantly higher in repeat breeders with subclinical endometritis. Thus, it could be assumed that TNFα and NO pathways are involved in repeat breeding in cows with subclinical endometritis.

TNFα is a cytokine produced by leukocytes infiltrating endometrial tissue, whereas iNOS is an inducible isotype of nitric oxide synthase secreted by macrophages (Hadded et al. 1995, 1997, Bondurant, 1999). Early embryonal mortality contributing to repeat breeding is driven by a complex network of many immunological active substances, among which TNFα and NO play a fundamental role. It is believed that an altered immune response, irrespective of the cause, has a cytotoxic effect on the embryo (Hadded et al. 1995, 1997). This working model, which links mechanisms of immunity with early pregnancy failure, has originated mainly from studies performed on rodent models.

To our knowledge, no such studies have been performed on bovine and other domestic animals under *in vivo* conditions The only available data on mice and rats showed enhanced endometrial/placental production of NO and TNFα in females with increased resorption rate of embryos (Haddad et al. 1995, 1997). In contrast, administration of a selective inhibitor of iNOS such as aminoguanidyne increased the average litter size (Haddad et al. 1995, Ogando et al. 2003). It has been hypothesized that also in cows, an inflammatory cascade can interfere with or inhibit endometrial preparation for implantation, triggering increased embryonal mortality (Gabler et al. 2009). The mRNA expression of selected cytokines and prostaglandins was significantly increased in the endometrium of cows with subclinical endometritis compared to healthy ones (Fischer et al. 2009, Gabler et al. 2009, Ghasemi et al. 2012). Although in those studies sampling was performed at an early time point (fourth week) after parturition, a similar uterine environment in the case of persistent inflammation could be anticipated during the breeding period.

It should be emphasized that the factors described above could explain just part of the general pathogenesis of repeat breeding, which is a very complex phenomenon. Although our study deals with a group of cows with subclinical endometritis, further comprehensive research on the immune response of the endometrium is needed to explain this problem in greater detail.

In conclusion, this study has demonstrated high prevalence of subclinical endometritis in repeat breeding cows and applicability of the cytobrush technique for its diagnosis. Moreover, possible involvement of TNFα and NO in the mechanism of repeat breeding in cows with subclinical endometritis has been indicated for the first time.

Acknowledgements

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