



Effect of Subclinical Endometritis on Pro-Inflammatory Cytokines, Prostaglandin-e2, MUC-1 and Cortisol Levels in Uterine Lavage of Repeat Breeder Dairy Cows

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Abstract | Subclinical endometritis (SCE) is considered one of the main causes of repeat breeding in cows leading to severe economic losses due to increased open days, calving interval and number of inseminations to achieve conception. The aim of the current study is to evaluate the uterine immunological and biochemical changes related to subclinical endometritis. Total number of sixty Holstein dairy cows, were divided into three groups equally, the first group was repeat breeder cows with subclinical endometritis (group-1) (SCE), second group was repeat breeder cows without subclinical endometritis (group-2) (No-SCE) (relying on cytobrush samples neutrophils polymorphnuclear cells (PMNs) %) while the third group was healthy cows (group-3) as negative control group. Our results revealed that uterine wash had a significant higher level of TNF- α , IL-10, PGE-2 and cortisol in SCE group compared to No-SCE and control groups ($P < 0.05$), however, MUC-1 level was significantly elevated in both SCE and Non-SCE groups compared to negative control group ($P < 0.05$). In conclusion, the elevated levels of pro-inflammatory cytokines, Prostaglandin-e2, MUC-1 and cortisol may share fundamentally in infertility disturbance associated with subclinical endometritis.

Keywords | Cytokines, Repeat breeder, Subclinical Endometritis, Infertility

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INTRODUCTION

Repeat breeder cow can be defined as a cow that has an apparent healthy condition but it does not conceive when bred for three or more consecutive times, either with a well-known fertile bull or artificially inseminated with excellent semen quality (Ahmadi and Dehghan, 2007; Warriachet al., 2008). Repeat breeder has been is considered a major problem in dairy herds worldwide (Bartlett et al., 1986). After parturition, uterus is exposed to several affections including clinical metritis, clinical endometritis and subclinical endometritis (Sheldon et al., 2006). Generally, endometritis is considered one of the most common reproductive diseases in dairy cows in postpartum period causing severe economic losses due to increased open days, calving intervals, and numbers of

services per conception (Salah and Yimer, 2017). Chronic uterine inflammation (Subclinical endometritis) affects approximately 37- 74% of parturient dairy cows (Gilbert et al., 2005; Linckee et al., 2007) and can be diagnosed by the presence of neutrophils polymorphnuclear cells (PMNs) threshold (10 % of cells in samples obtained from endometrium by cytobrush in absence of any clinical signs of Endometritis) (Janowskiet al., 2013; Butt et al., 1993). Bacterial infection causing uterine disease is the corner stone of post-partum infertility (Sheldon and Dobson, 2004). The most common causative bacterial species in occurrence of uterine infection are *AC pyogenes*, *Fusobacterium necrophorum*, *Escherichia coli* and *Bacterioides melaninogenicus* (Studeret and Morrow, 1978; Olson et al., 1986; Noakes et al., 1989; Bonnett et al., 1991; Sheldon et al., 2009). Bacterial lipopolysaccharide (LPS) stimulate the

secretion of pro-inflammatory cytokines as tumor necrosis factor alpha (TNF- α) and chemokines (e.g. IL-8) which stimulate chemoattraction of neutrophils and monocytes and their diapedesis to the site of infection and enhance their phagocytosis (Butterfield et al., 2006; Singh et al., 2008). Increasing the expression levels of pro-inflammatory cytokines in uterine endometrium tissue and/or in serum consider as a diagnostic and prognostic indicator of the development of endometritis in cows (Galvao et al., 2011; Ghasemi et al., 2012; Islam et al., 2013; Kasimanickam et al., 2013). Bacterial LPS switch the endometrial prostaglandin production from prostaglandin F₂-alpha (PGF-2a) to prostaglandin E₂ (PGE-2) (Sheldon et al., 2009) to control the inflammation (Sugimoto and Narumiya, 2007) however, PGE is a leuteotropic substance which enhance corpus luteum (CL) persistence in ruminant leading to disturbance in normal reproductive cycle (Pratt et al., 1979; Chenault, 1983; Poyser, 1995). In addition, endometrial surface epithelial antimicrobial peptides as mucin-1 (MUC-1) play an important role in defense against microbial infection and their levels are increased during endometrial affections (Davies et al., 2008). Likewise, corticosteroids (e.g. adrenal cortisol) play an important role as an anti-inflammatory response to any inflammatory state in the animal body (Cronstein et al., 1992), while their deleterious impact on fertility and reproduction is well-known (Daley et al., 1999). Therefore, this study was performed to evaluate the effect of subclinical endometritis on some immunological (TNF- α , IL-10 and MUC-1) and biochemical factors (PGE-2 and cortisol) in uterine wash of repeat breeder cows, which may help in understanding the role of these factors in relation to low reproductive performance of these affected cows.

MATERIAL AND METHODS

EXPERIMENTAL DESIGN

Lactating multiparous Holstein cows (n =60), 4-6 years old and weighting approximately 500 kg from several dairy farms in desert Alexandria/Cairo road, Egypt between July 2017 and May 2018 were selected to perform this study. All of the selected animals were almost in luteal phase and the case history of these cows was reviewed carefully to make sure that they did not receive any antibiotic or reproductive hormones treatment within last 21 days before clinical examination and samples collection. Forty cows were considered as repeat breeder because of failing to re-conceive after three or more consecutive inseminations with trusted quality semen straws after last parturition without any apparent abnormal clinical signs, which subdivided into two groups; (20 each). Group 1 (SCE) represents repeat breeder cows with subclinical endometritis depending on PMNs% upon cytobrush cytological uterine samples examination, group 2 (No-

SCE) represents repeat breeder cows without subclinical endometritis and finally group 3 (negative control) has twenty reproductively healthy, normally cycling cows, as they were inspected externally, transrectally, ultrasonically and cytologically using cytobrush for presence of any abnormal findings.

SAMPLING, ENDOMETRIAL CYTOLOGY AND ELISA

Samples were collected using cytobrush (Gobal Surgimed Industries, India), the cytobrush was modified to be used in cow by its fixation to stainless steel rod (Artificial insemination gun, 60 cm length) and inserted trans-vaginally through stainless steel tube about 50 cm length. Vagina was lubricated (AQUAGEL Lubricating Jelly, Ecolab, USA), for easy insertion of the instrument which was directed trans-rectally to pass through cervix to reach uterine horn. Cytobrush was rotated in a clockwise manner against uterine wall and retracted before instrument removal to obtain the sample. This instrument was kept sterile between usages (steam-sterilization for 5 min). Immediately after retraction, cytobrush was rolled on to glass slide, air dried and fixed using methanol for 5 min then stained using Giemsa stain (Oxford Laboratory Reagent, India) for 20 min. After dryness, the slides were examined microscopically (Leitz, Germany) at X400 at which approximately 100 cells were counted to detect the percent of neutrophils (PMNs %) for quantitative assessment of endometrial inflammation. Endometrial threshold value of 10% PMNs was used for diagnosis of subclinical endometritis (Janowski et al., 2013). Uterine lavage was conducted through infusion of 50 ml of sterile saline solution into uterus using intrauterine stainless steel catheter with a syringe inserted into the uterus followed by rectal massage to collect the uterine wash fluid. The collected fluid was centrifuged at 3000 x g for 10 min at 4°C for clarification; the supernatant were kept at -80°C until analysis (Brodzki et al., 2015) for detection of the following biomarkers levels using specific ELISA kits; TNF- α , IL-10, PGE-2 (Abcam, USA) and, cortisol (Abnova, Taiwan) and Mucin-1 (CUSABIO, China). Absorbance readings (OD) were performed using an automatic micro-plate reader (Stat Fax, USA).

STATISTICAL ANALYSIS

All values were expressed as means \pm SE. Statistical analysis was performed using the SPSS statistical package v 22.0 for Windows (IBM, Armonk, NY, USA). Data were checked for normality using Shapiro-Wilk test. Mean values were compared between different groups using one-way analysis of variance (ANOVA) followed by post-hoc multiple comparisons Duncan's test. P-value <0.05 was considered statistically significant.

The levels of TNF- α , IL-10, PGE-2 and cortisol in uterine wash were significantly elevated ($P < 0.05$) in repeat breeder animals with subclinical endometritis (SCE) in comparison to repeat breeder animals without subclinical endometritis (No-SCE) and healthy negative control animals (Table 1), while their levels did not record any significant changes ($P < 0.05$) in No-SCE group compared to healthy negative control animals (Table 1). Whereas the level of MUC-1 was significantly higher ($P < 0.05$) in both of repeat breeder animals with subclinical endometritis (group 1) and those without subclinical endometritis (group 2) than healthy animals (negative control group) (Table 1); however, its level was significantly much higher ($P < 0.05$) in group 1 than group 2 (Table 1).

Table 1: Levels of TNF- α , IL-10, PGE-2, MUC-1 and cortisol in uterine wash of repeat breeder animals groups (without and without subclinical endometritis) in compare to negative control healthy cows.

Group (n=20/group)	Group 1 (SCE)	Group 2 (No-SCE)	Group 3 (Healthy)
Evaluated parameters			
TNF- α (pg/ml)	551.10 \pm 30.81 ^a	138.07 \pm 14.35 ^b	133.31 \pm 13.58 ^b
IL-10 (pg/ml)	131.00 \pm 8.47 ^a	49.26 \pm 4.53 ^b	43.40 \pm 4.04 ^b
PGE-2 (ng/ml)	37.63 \pm 3.25 ^a	12.35 \pm 1.14 ^b	10.86 \pm 0.84 ^b
MUC-1(U/ml)	115.80 \pm 7.56 ^a	71.88 \pm 9.15 ^b	25.60 \pm 2.94 ^c
Cortisol (ng/ml)	11.22 \pm 0.84 ^a	3.14 \pm 0.32 ^b	2.72 \pm 0.33 ^b

All values are expressed as means \pm SE; Means within the same row of different litters are significantly different at ($p < 0.05$); SCE: repeat breeder cows affected with subclinical endometritis; No-SCE: repeat breeder cows not affected with subclinical endometritis. TNF- α : tumor necrosis factor-alpha; IL-10: Interleukin-10; PGE-2: prostaglandin-2; MUC-1: mucin-1.

DISCUSSION

Subclinical endometritis is a wide spread problem in most of dairy farms (Parkinson, 2009; Salasel et al., 2010) and it can be defined as inflammation of endometrium that results in considerable decrease in reproductive performance of the affected animal (affected animals take longer to conceive with lowered conception rate) without any signs of clinical endometritis (Salah and Yimer, 2017). The percentage of PMNs % in uterine samples obtained by cytobrush or uterine flushing is the key diagnostic method for presence of subclinical endometritis (Kasimanickam et al., 2004; Santos et al., 2009), as the inflammatory cytokines and chemokines are produced in response to the presence of the bacterial lipopolysaccharide (LPS) which attract neutrophils and stimulate their influx within uterine lumen in order to eliminate causative bacteria (Sheldon et al., 2009). Previous studies might explain the

significant increase in TNF- α levels in uterine lavage of the affected animals with subclinical endometritis, as TNF- α is a members of pro-inflammatory cytokines which is produced in response to infection and/or inflammation to promote phagocytic cells diapedesis, chemoattraction and phagocytosis (Butterfield et al., 2006; Singh et al., 2008; Kim et al., 2014). Collectively, inflammatory cytokines (including TNF- α) may impair steroidogenesis by follicular granulosa cells (Spicer and Alpizar 1994; Sheldon et al., 2009) and specifically, TNF- α has been proved to have a great role in occurrence of the early embryopathy (Pampfer et al., 1997) as the inflammatory cascade may inhibit or interfere with endometrial preparation for embryo implantation (Gableret al., 2009). IL-10 is produced from a variety of T-cells and has been proved to have an anti-inflammatory activity to protect the uterine tissues from overly virulent activity of inflammatory cells and mediators via its role with the regulatory suppressor T CD8+ cells (Stumhofer et al., 2007; Tangiet al., 2005; Askenasy et al. 2008; Brodzkiet al., 2014b) and this may declare the reason of its significant increase in uterine washings of subclinical endometritis-affected cows (Brodzki et al., 2014a; Kim et al., 2014). However, IL-10 is proved to be responsible for weakening of uterine local resistance, persistence of post-partum infection and inflammation (Brodzki et al., 2015), these reasons collectively may explain the impacts of these inflammatory mediators (TNF- α and IL-10) on fertility which result in altered reproductive performance (including repeat breeder) in animals suffering from subclinical endometritis. The significant increase in PGE-2 in uterine wash of cows with subclinical endometritis (SCE group) may be attributed to the role of PGE-2 in inflammation control through binding with prostaglandin E receptors 2 and 4 (PGER2 and PGER4) (Herath et al., 2006; Sugimoto and Narumiya, 2007) which can increase the production of anti-inflammatory IL-10 (Demeure et al., 1997). PGE-2 is luteotropic in ruminants (Poyser, 1995) which may extend luteal phase and favor luteal maintenance resulting in impaired fertility, disturbance in PGF-2a due to switching of endometrial gland production from PGF-2 alpha to PGE-2 (Manns et al., 1985). Mucin-1 (MUC-1) is a glycosylated transmembrane protein which is secreted by epithelial cell and may have a role in defense of endometrium against microbial infections (Brayman et al., 2004) and this may illustrate its increment in uterine lavage of subclinical endometritis-affected repeat breeder cows. Our results were in agreement with Kasimanickam et al. (2014) who proved the increase in MUC-1 expression levels with uterine disease. MUC-1 level increase in uterine wash of repeat breeder cows of group 2 (not affected by subclinical endometritis) may be owed to their failure to conceive for long period as the concentration of MUC-1 was decreased in uterus of normally cycling individuals only nearing conception to facilitate its occurrence (Carson

et al., 1998). The role of corticosteroids as adrenal naturally produced cortisol in relieving of inflammatory conditions was well discussed by Cronstein et al. (1992), but the exposure to chronic inflammatory state (as subclinical endometritis) may cause glucocorticoids receptors resistance which may result in failure of cortisol to down-regulate inflammatory response (Cohen et al., 2012). Also, cortisol may impair follicular development and ovulation in ruminant (Daley et al., 1999; Macfarlane et al., 2000), which might enhance reproductive insufficiency of such animals.

CONCLUSION

Our study revealed that the elevated levels of pro-inflammatory cytokines (TNF- α and IL-10), Prostaglandin-e2, MUC-1 and cortisol may share fundamentally in infertility disturbance associated with subclinical endometritis in dairy cows.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

AUTHORS CONTRIBUTION

All authors contributed equally in this work.

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