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; Warriach et al., 2008). Repeat breeder has been 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; Lincke 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) (Janowska et al., 2013; Butt et al., 1993). Bacterial infection causing uterine disease is the cornerstone of post-partum infertility (Sheldon et al., 2009). The most common causative bacterial species in occurrence of uterine infection are *AC pyogenes*, *Fusobacterium necrophorum*, *Escherichia coli* and *Bacterioides melaninogenicus* (Studer et al. 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 chemotraction 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 pro-inflammatory 

**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 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).

**MATERIAL AND METHODS**

**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 ± 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 control animals (Table 1), while their levels did not record any significant changes (P > 0.05) in No-SCE group compared to healthy 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.

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

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 member of pro-inflammatory cytokines which is produced in response to infection and/or inflammation to promote phagocytic cells diapedesis, chemoaattraction 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 prostataglandin 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-2a 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., 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
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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