



Subclinical endometritis increases oxidative stress and modulates polymorphonuclear leukocyte functions in crossbred cows

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ABSTRACT

Subclinical endometritis (SCE) adversely affects fertility and is a diagnostic challenge in bovine practice. In the present study, it was hypothesized that SCE in cows influence the plasma levels of nitric oxide (NO), lipid peroxide (LPO) and polymorphonuclear (PMN) cell functions. Cows with SCE (n,12) were selected on the basis of positive colour reaction of cervico-vaginal mucus (CVM) to Whiteside test, alkaline pH of CVM and presence of > 5% PMN cells in uterine cytology smears. Cows without endometritis served as negative control (n,12). Functions of PMN cells were assessed by estimating superoxide (O_2^-) and hydrogen peroxide (H_2O_2) production ability. Further, to assess the inflammatory status and oxidative stress, plasma levels of NO and LPO were measured. The results revealed that cows with SCE had significantly higher H_2O_2 (19.70 ± 6.43 vs 2.52 ± 0.71 nmol/ 2×10^6 cells/30 min incubation) in isolated blood PMN cells as compared to non endometritic cows ($P < 0.05$). Similarly, an increased plasma concentrations of NO (81.34 ± 1.70 vs 57.50 ± 1.36 μ mol/L) and LPO (712.00 ± 50.39 vs 402.78 ± 21.61 nmol MDA/L) were observed in cows with SCE. The results suggested that SCE increases oxidative stress and PMN cell functions despite being a local inflammation and may have potential in the diagnosis of SCE or monitoring the efficacy of treatment.

Key words: Cows, Lipid peroxide, Nitric oxide, PMN cell function, Subclinical endometritis

Subclinical endometritis (SCE), the inflammation of endometrium in the absence of any clinical signs (Sheldon *et al.* 2009), is difficult to diagnose and usually results into conception failure or embryonic mortality leading to a significant increase in the incidence of repeat breeding syndrome (Agarwal *et al.* 2005). Abnormal calving, retention of fetal membranes and postpartum uterine infections increase risk of SCE in dairy animals (Salasel *et al.* 2010). After recognising pathogen, the immune cells release pro-inflammatory molecules and nitric oxide (Baumann and Gauldie 1994). Moreover, lipid peroxidation is capable of causing irreversible damage to biomolecules in different stressful conditions. Inflammatory condition and infection increase the production of reactive oxygen species (ROS). Higher ROS production in the cells leads to imbalance between oxidants and antioxidants (Lykkesfeldt and Svendsen 2007) resulting in oxidative stress. Cytotoxic malonyldialdehyde (MDA), a by-product of lipid peroxidation, is an indicator of oxidative stress (Placer *et al.* 1966).

Polymorphonuclear cells (PMNs) are the earliest and

most important phagocytic cell type recruited from the peripheral circulation to the uterine lumen in response to pathogen challenge (Subandrio *et al.* 1997). Phagocytic activity of PMNs in peripheral blood and uterine lumen remains similar, although somewhat less at uterine lumen and therefore, activity of PMNs in peripheral blood may be a gross estimation of uterine neutrophils function (Anderson *et al.* 1985). The impaired PMNs function prior to calving was reported to be a major risk factor in the development of SCE (Hammon *et al.* 2006). However, PMNs function has not yet been studied in cows with SCE. In the present study, it was hypothesised that SCE influence the plasma levels of nitric oxide, lipid peroxide and alters the PMNs function in the peripheral blood of crossbred cows.

MATERIALS AND METHODS

This study was conducted at Livestock production and management (Cattle and Buffalo farm) Section, IVRI, Izatnagar. Experimental crossbred cows (n=24) were over 60 days postpartum, second to fifth parity and had good body condition. The cows were maintained under iso-managerial conditions. Subclinical endometritis (n = 12) was diagnosed at estrus on the basis of alkaline pH of the cervico-vaginal mucus (CVM) (Tsiliogianni *et al.* 2001), positive colour reaction of CVM to white side test (Popov-

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Y 1969) and presence of more than 5% neutrophils in uterine cytology (Gilbert *et al.* 2005). Cows that were negative for SCE formed control group (n=12). Peripheral blood (10 ml) was collected in heparinised (20 IU/ml of blood) sterilized disposable centrifuge tubes. To study the interestrus variability and to decrease the effect of sampling and subjective errors, blood sampling was done on two consecutive estrus periods. Nitric oxide (NO) and lipid peroxide (LPO) levels were estimated in the blood plasma. Blood polymorphonuclear (PMN) cell functions were studied by measuring the superoxide and hydrogen peroxide production that indicates the respiratory burst activity.

Nitric oxide assay: Nitric oxide concentration in plasma was estimated as stable nitrite by modified Greiss reaction (Sastri *et al.* 2002). Results were expressed as $\mu\text{mol/L}$ of plasma from the standard curves.

Lipid peroxidation assay: Malonyldialdehyde (MDA) level, an index of lipid peroxidation was measured by the double heating method of Draper and Hadley (1990). The concentration of MDA was calculated based on the absorbance coefficient of the TBA-MDA ($\epsilon=1.56 \times 10^5 \text{ cm}^2/\text{M}$).

Polymorphonuclear cells (PMNs) function assay: Respiratory burst activity of PMN(s) was estimated on the basis of superoxide and hydrogen peroxide production ability of PMN cells. The PMN cells were separated from blood and viable cell count of isolated blood PMN cells were determined by Trypan blue exclusion technique as per method of Colligan *et al.* (1994). The cell concentration in PMN suspension was adjusted at $1.0 \times 10^7 \text{ cells/ml}$ following the method of Gentle and Thompson (1990).

Superoxide production assay: The superoxide production by PMN cells was measured by nitroblue tetrazolium (NBT) reduction assay as per Nagahata *et al.* (1988) with slight modification.

Hydrogen peroxide production assay: The production of hydrogen peroxide (H_2O_2) was assayed in blood PMNs as per Pick and Keisari (1980). Results were expressed as nanomoles of H_2O_2 per 2×10^6 PMN cells.

Statistical analysis: The means of NO, LPO, superoxide and H_2O_2 in SCE and non-SCE crossbred cows were compared by independent t-test using SPSS version 17. Confidence level was set at 95%.

RESULTS AND DISCUSSION

Results of the various biochemical parameters are shown in Table 1. Subclinical endometritic cows had greater plasma concentration of NO and LPO as well as H_2O_2 production than non-SCE cows. However, mean superoxide production in isolated blood PMN cells did not differ between SCE and non-SCE cows. To our knowledge, the current study represents the first investigation of the oxidative stress and polymorphonuclear leucocyte functions in cows suffering from subclinical endometritis. The results of the present study are in accordance with Li *et al.* (2010), who reported higher levels of NO in the blood and uterine fluids as well higher expression of nitric oxide synthetase-2 (NOS2) in uterine biopsy of cows with subclinical and clinical endometritis, however, the values in our study were slightly higher. Li *et al.* (2010) reported a mean NO concentration in blood was $8.15 \pm 0.97 \mu\text{mol/L}$ in subclinical endometritis and $8.77 \pm 1.35 \mu\text{mol/L}$ in clinical endometritis in comparison to $6.43 \pm 0.93 \mu\text{mol/L}$. In addition, Blum *et al.* (2000) also found an increased concentration of plasma and milk nitrite and nitrate after intramammary infusion of *E. coli* bacteria or endotoxin. NO being an important mediator of inflammation, the concentrations are higher in various inflammatory conditions (Sharma *et al.* 2007). However, interestrus variability was observed only for NO in cows with SCE which might be due to aggravation of oxidative stress in cows with SCE.

Endometritis reportedly damage the endometrial health and function leading to infertility conditions in domestic animals (Williams *et al.* 2007a). McDougall *et al.* (2011) reported that cows with SCE had a lower proportion (0.57 vs 0.97) pregnant overall, decreased pregnancy to first service, and took longer to conceive (56.1 vs 32.6 days from the planned start of mating), compared with cows without the condition. Uterine endometrial epithelial and stromal cells play a key role in detecting *E. coli* in the reproductive tract because they express the TLR4/CD14/MD-2 complex required for LPS recognition (Herath *et al.* 2006, Patra *et al.* 2013). Endometrial cells respond to bacteria by activation of TLRs and subsequently stimulate the production of proinflammatory cytokines, chemokines, and prostaglandins (Schaefer *et al.* 2004, Herath *et al.* 2006). LPS stimulated endometrial cells express numerous

Table 1. Concentrations (mean $\pm$ SEM) of plasma nitric oxide, lipid peroxide, production of superoxide and hydrogen peroxide in polymorphonuclear leukocytes in crossbred cows with or without subclinical endometritis (SCE).

Parameter	Sampling	Non-SCE (n=12)	SCE(n=12)	P value
Nitric oxide ($\mu\text{mol/L}$)	Estrus	57.50 $\pm$ 1.36	81.34 $\pm$ 1.70 ^a	P<0.05
	Subsequent estrus	55.50 $\pm$ 0.88	107.33 $\pm$ 3.51 ^b	P<0.05
Lipid peroxide (nmol/L MDA)	Estrus	402.78 $\pm$ 21.61	712.00 $\pm$ 50.39	P<0.05
	Subsequent estrus	409.18 $\pm$ 14.75	759.62 $\pm$ 49.95	P<0.05
Superoxide ($\Delta\text{OD}/2 \times 10^6 \text{ cells}/30 \text{ minute incubation}$)	Estrus	0.15 $\pm$ 0.03	0.22 $\pm$ 0.06	NS
	Subsequent estrus	0.19 $\pm$ 0.02	0.30 $\pm$ 0.14	NS
Hydrogen peroxide (nmol/ $2 \times 10^6 \text{ cells}/30 \text{ min incubation}$)	Estrus	2.52 $\pm$ 0.71	19.70 $\pm$ 6.43	P<0.05
	Subsequent estrus	2.35 $\pm$ 0.45	25.92 $\pm$ 7.65	P<0.05

NS, nonsignificant; a:b, significant (P<0.05) difference between estrus and subsequent estrus.

proinflammatory cytokines and chemokines, including tumour necrosis factor- α , nitric oxide, interleukin-1 (IL-1), IL-6 and IL-8 in uterus of cattle and buffalo (Herath *et al.* 2009, Fischer *et al.* 2010, Loyi *et al.* 2013).

In this study, the plasma LPO concentration increased significantly in cows with SCE as compared to normal non-endometritic cows. This receives support from Kizil *et al.* (2010) who reported a higher LPO levels in cows with acute puerperal metritis than healthy ones. Ranjan *et al.* (2005) also reported an enhanced level of erythrocyte lipid peroxides, which suggested oxidative stress in animals with subclinical and clinical mastitis. Moreover, Atroshi *et al.* (1989) reported an increase in free fatty acids and lipid peroxidation in mastitic bovine milk. In experimental mastitis induced by intramammary infusion of *E. coli* bacteria, a variable oxidative stress was also noticed (Weiss *et al.* 2004).

The mean superoxide production in isolated blood PMN cells did not differ between SCE and non-SCE cows. However, H_2O_2 production was found to be significantly higher in cows with SCE than non-SCE both at first and subsequent estrus. The increased respiratory burst activity in subclinical endometritic cows might be attributed to the stimulation of mature neutrophils by bacterial endotoxins (LPS) or other inflammatory mediators to release ROS. Heyneman *et al.* (1989) reported that cows with mastitis had increased PMN cell functions. However, impaired PMNs functions were observed in periparturient cows with mastitis, endometritis and metritis (Hammon *et al.* 2006).

Bovine blood PMN cells have the potential to produce ROS to eventually kill the engulfed bacteria. The oxygen dependent mechanism of blood PMNs is based on its respiratory burst resulting in oxygen consumption and glucose oxidation through hexose monophosphate shunt leading to augmented production of superoxide and hydroxyl radicals (Tizard 2009). Our results revealed a higher H_2O_2 production from blood PMN cells in subclinical endometritic cows that might be attributed to the ability of stimulated neutrophils by bacterial endotoxin (LPS) or other inflammatory mediators to release ROS. Mehrzad *et al.* (2002) reported that the production of ROS is one of the most important killing mechanism of primed PMNs. Pande *et al.* (2013) observed that endometritis leads to an oxidative stress at the ovarian level, thereby hampering the ovulatory capability and steroidogenesis.

It can be concluded that SCE increased oxidative stress and PMN cell functions despite being a local inflammation and may have potential in the diagnosis of SCE or monitoring the efficacy of treatment in cows.

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